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13. SUPPLEMENTARY NOTES

14. ABSTRACT Active military personnel and veterans represent a large population of individuals suffering from chronic pain due to multiple types of traumatic injuries, including spinal cord injury. In fact, chronic pain affects up to 80% of patients who have sustained a spinal cord injury. Unfortunately, existing treatments for chronic pain are usually ineffective, greatly worsening what is already the substantial burden of injury. Our research program aims to identify the basic causes of chronic pain, and to develop new treatments for chronic pain based on the powerful biology of neural stem cells. We recently discovered that neural stem cells, grafted into the injured spinal cords of rats and monkeys, exhibit a remarkable ability to form large numbers of new connections over very long distances in the host spinal cord. Even after complete severing of the spinal cord, neural stem cells form new relays across the injury that support partial recovery of function in rat models. This work is in now in translational development that could lead to human clinical trials in the near future. A consideration of great importance in this work is whether transplants of neural stem cells after spinal cord injury will improve, stabilize or worsen traumatically induced chronic pain. This information is essential for the initiation of human clinical trials, and is consistent with our overarching research vision to understand the causes and develop effective treatments for chronic pain. **This project will foster work in a new direction in spinal cord injury research while addressing a neglected issue that extracts an enormous human toll from our injured soldiers and veterans: chronic pain. Successful completion of this research program will add to our basic knowledge of cellular and molecular causes of pain, and could lead to the clinical implementation of an innovative new therapy for chronic pain based on the powerful biology of stem cells.**

15. SUBJECT TERMS

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INTRODUCTION: Active military personnel and veterans represent a large population of individuals suffering from chronic pain due to multiple types of traumatic injuries, including spinal cord injury (SCI). Chronic pain so greatly affects quality of life that depression and suicide frequently result. Recently, we found that multi-potent neural progenitor cell (NPC) grafts fill the lesion site in severe SCI (complete transection) and extend large numbers of axons over long distances through the lesioned host spinal cord, forming novel neural relays that support electrophysiological conduction across the lesion site and generate functional recovery (Lu *et al*., Cell, 2012). Furthermore, *human* neural stem cells and induced pluripotent stem cells (iPSCs) isolated from a normal, 86-year-old human and driven to NSC fate grafted into immunodeficient rats, also send out large numbers of axons over long distances in the injured rat spinal cord (Lu *et al*., Neuron, 2014). Given the impact of these findings, the work is in translational development. However, it remains unclear whether strategies that seek to regenerate the spinal cord or improve spinal cord function following SCI can mitigate or exacerbate chronic SCI pain. One of the impediments to understanding the causes of chronic SCI pain includes the availability/use of preclinical rodent models that accurately reflect the human condition. We will use severe T3 SCI models, since 1) the majority of patients with chronic SCI pain result from severe injuries typically at the cervical and upper thoracic levels; and 2) our data shows that in severe SCI, NPCs facilitate axon growth over long distances and support functional motor recovery. Yet, whether sensory systems are altered remains unclear. Our studies will reveal novel insights to the pathophysiology of chronic SCI pain and whether NPCs can modify pain outcomes. This proposal will test whether neural progenitor cells implanted into the severely lesioned adult spinal cord, will modify post lesion molecular sprouting responses to attenuate chronic SCI pain.

KEYWORDS: Neural stem cells, spinal cord injury, neuropathic pain, spasticity, allodynia, hyperalgesia, regeneration, operant models, RNA transcriptome, dorsal root ganglia

ACCOMPLISHMENTS: What were the major goals of the project?

Identify Pain Related Behaviors and Cellular/Molecular Outcomes in Rats with Severe SCI

Examine Effects of Neural Progenitor Cell Grafts on Pain Related-Behaviors and Molecular Mechanisms after Severe SCI

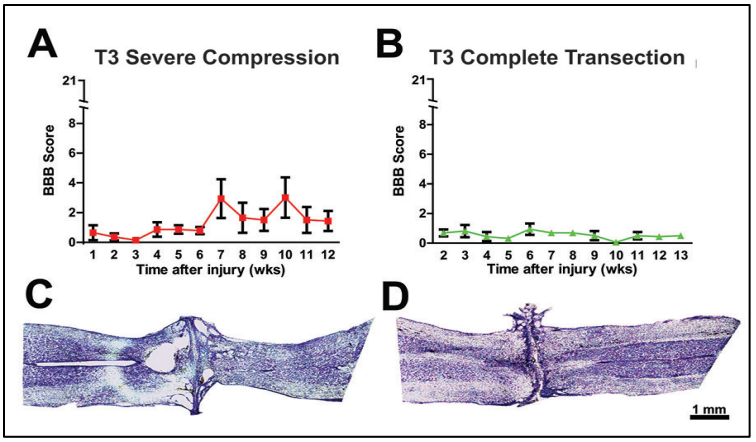


Fig. 1. Comparison of BBB scores in models of severe SCI. ***A.*** T3 severe compression (n=7). ***B.*** T3 complete transection (n=8). Data are expressed as mean ±SEM. ***C.*** Terminal lesion histology shows a band of disrupted parenchyma with associated cavitation in rats with T3 severe compression. ***D***. Rats with T3 compression showed a narrow lesion surrounded by inflammatory cell infiltrates. Horizontal sections through the lesion site are oriented rostral to caudal from left to right. Scale bar is 1mm.

Perform Transcriptome Analysis on DRG neurons in Rats with Severe SCI

What was accomplished under these goals?

Specific Objective: Perform T3 complete transection, T3 severe compression and sham surgeries in F344 rats and access pain related behaviors by evoked, spontaneous and operant models (PEAP). Naïve animals serve as controls.

These activities includes work proposed in the Major Task 1 (Subtasks 1 and 2) of the Statement of Work (SOW). As outlined in the SOW, we performed T3 complete transection, T3 severe compression surgeries in F344 rats. Naïve and sham animals served as controls. Both T3 severe compression and T3 complete transection resulted in extensive loss of hindlimb function that was associated with a score <3 on the 21 point BBB locomotor scale (**Fig. 1A,B**). In rats with T3 severe compression, BBB scores rose slightly beginning 7 weeks following injury (**Fig. 1A**). Nonetheless, deficits in both groups remained severe and did not differ substantially. T3 severe compression lesion histology demonstrated a band of disrupted parenchyma across the compression site with partial surrounding cavitation (**Fig. 1C**). T3 complete transection injuries are characterized by a narrow lesion surrounded by inflammatory cell infiltrates (**Fig. 1D**). Lesion histology confirmed the absence of spared tissue in the lesion site of all rats included in the study.

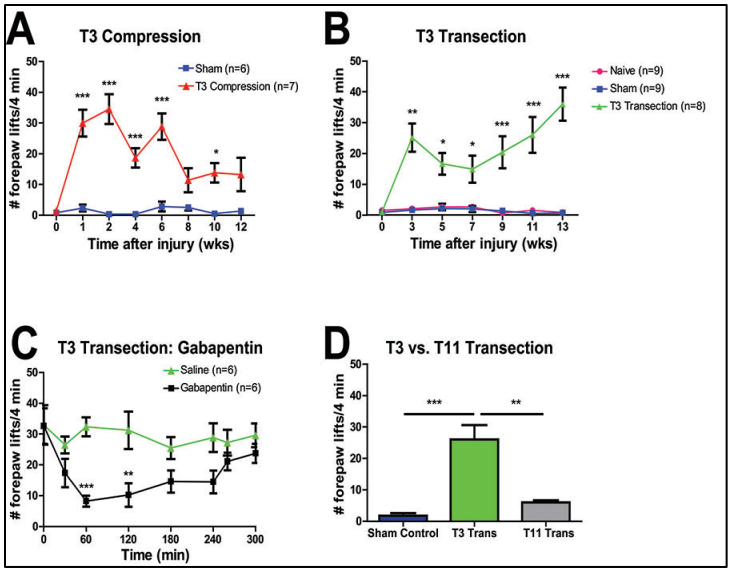


Fig. 2. T3 severe SCI induces forepaw spontaneous pain-related behaviors in rats with A. T3 severe compression compared to sham control. **B**. T3 complete transection compared to naïve and sham control. Graphs show the number of forepaw lifts within 4 minutes over 13 weeks. N=6-9/group. *p<0.05, **p<0.01, ***p<0.001 compared to sham control and naive by two-way repeated measures ANOVA with Bonferroni *post-hoc* analysis. **C**. Effect of gabapentin (30 mg/kg i.p.) on spontaneous forepaw lifts when administered at 13 weeks after T3 complete transection. N=8/group. **p<0.01, ***p<0.001 compared to saline treatment by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* analysis. **D**. Effect of spinal level of injury on spontaneous forepaw lifts 6 weeks after injury in sham control , T3 complete transection (T3 Trans) and T11 complete transection (T11 Trans). N=4/group. **p<0.01, ***p<0.001 by one-way ANOVA followed by Tukey's *post-hoc* test. Data are expressed as mean ± SEM.

Once surgeries were successfully executed, we began to determine the types of pain modalities exhibited in severe SCI rats (subtask 2). Initially, we tested spontaneous pain related behaviors. Spontaneous pain is a prominent manifestation of at-level pain reported by SCI patients (Baastrup et al., 2012). To determine whether spontaneous

pain was present in rats that have sustained severe SCI, we evaluated spontaneous paw lifting in the forelimbs. Forelimb measures represent responses from neural circuitry located at least two spinal segments above the lesion, and could result from plastic rearrangements evoked by the proximity to the lesion site. In control rats, spontaneous forepaw lifting behavior was negligible (fewer than 3 lifts/4 min) and remained unchanged over the duration of the study (**Fig. 2A,B**). In contrast, rats with either T3 severe compression (**Fig. 2A**) or T3 complete transection (Fig. 2B) exhibited significant spontaneous forepaw lifting over 12-13 weeks compared to controls (repeated measures ANOVA, $p<0.01$, comparing T3 lesioned groups to their respective control groups). In rats with T3 severe compression, spontaneous forepaw lifting declined 8 weeks after injury ($p<0.05$ comparing Wk 8, 10 and 12 to Wk 2 by one-way repeated measures ANOVA followed by Tukey's test), whereas these behaviors increased over time in rats with T3 complete transection. The reduction in spontaneous forepaw lifting was not linked to variability of locomotor outcome as spontaneous lifting behavior in the three rats with BBB scores greater than 3 were not significantly different from those of the four rats with BBB scores less than 3 (by unpaired two-tailed t-test comparing average weeks 8-12 spontaneous forepaw lifts and linear regression). Notably, at-level pain sensations in human patients of SCI comprise abnormal evoked responses such as allodynia in response to light touch and cold hypersensitivity (Baastrup et al., 2012). Thus, we examined both tactile and cold forelimb allodynia in rats with severe SCI. Sham control and naïve rats had a 50% forepaw withdrawal threshold that consistently remained

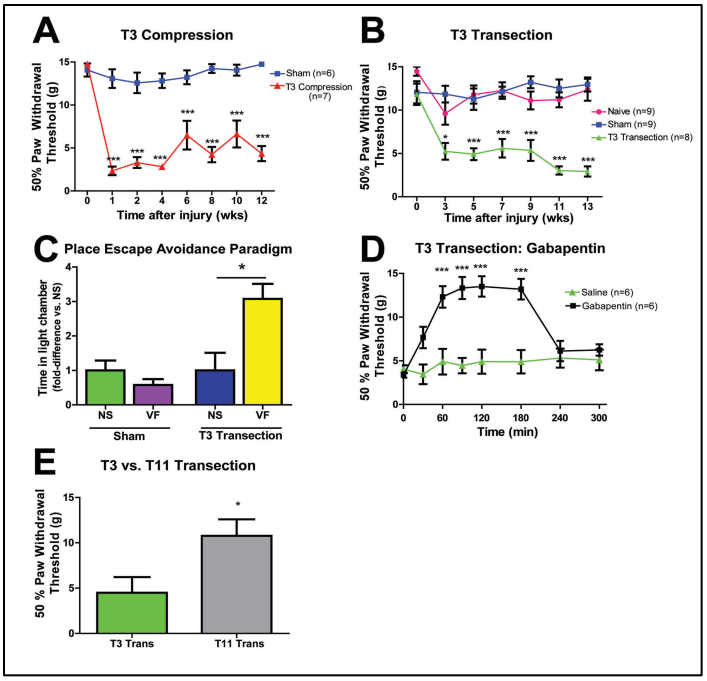


Fig. 3. Forepaw tactile allodynia develops after severe SCI. ***A.*** T3 severe compression compared to sham control. ***B.*** T3 complete transection compared to naïve and sham control. Forepaw tactile withdrawal thresholds were evaluated in rats over 13 weeks. N=6-9/group. *p<0.05, **p<0.01 ***p<0.001 compared to sham control and naive by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* analysis. Data are expressed as mean ± SEM. ***C.*** Place escape avoidance paradigm (PEAP) conducted 4 weeks after sham or T3 complete transection surgery. Data are expressed as "fold-difference" compared to non-stimulated (NS) N=5/group. *p<0.05 compared to NS by unpaired, two-tailed t-test. ***D.*** Effect of gabapentin (30 mg/kg i.p.) on forepaw tactile withdrawal when administered at 4 weeks after T3 complete transection (n=6/group). ***E.*** Effect of spinal level of injury on tactile withdrawal 10 weeks after injury in T3 complete transection (T3 Trans) and T11 complete transection (T11 Trans). N=4/group. ***p<0.01 by one-way ANOVA followed by Tukey's *post-hoc* test.

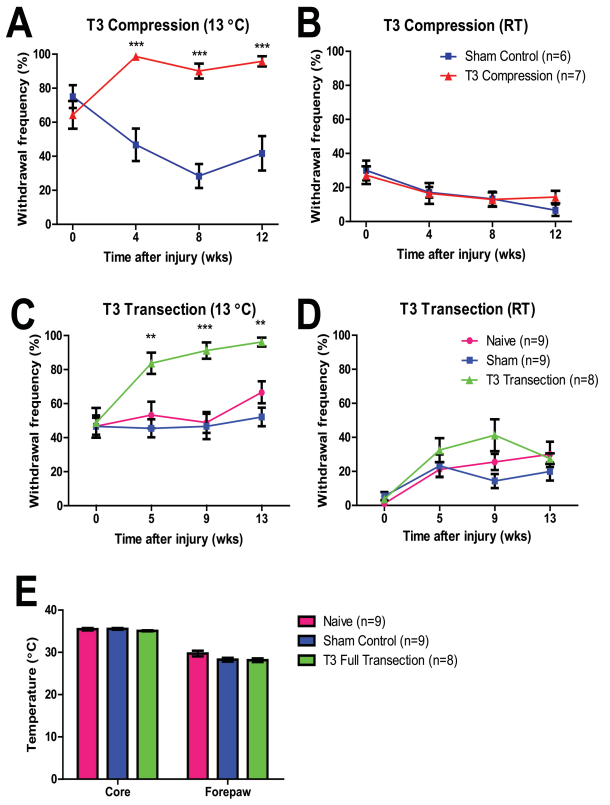


Figure 4

Fig. 4. Cold allodynia develops in the forepaws after T3 severe SCI. ***A.*** T3 severe compression in response to the probe held at 13 °C, compared to sham control. ***B.*** T3 severe compression in response to the probe held at room temperature (RT). ***C.*** T3 complete transection in response to to the probe held at 13 °C, compared to naïve and sham control. ***D.*** T3 complete transection in response to the probe held at RT. Sensitivity to cold was assessed by withdrawal frequency in response to contact with at 13 °C Peltier probe. N=6-9/group. **p<0.01, ***p<0.001 compared to sham control and naive by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* analysis. ***E.*** Core and forepaw surface temperature in naïve, sham control and rats with T3 complete transection 13 weeks following injury. N=8-9/group. No significant difference between groups by one way ANOVA. Data are expressed as mean ± SEM.

in the non-allodynic range (>10g) over the duration of the study (**Figs. 3A,B**). However, rats with T3 severe compression exhibited consistently reduced 50% tactile withdrawal threshold beginning 1 week post-injury and continuing through the end of the study 12 weeks later (**Fig. 3A**). Every rat had an average 50% paw withdrawal value less than 6.5 g over the 1-12 week period, indicating consistent development of allodynia. Rats with T3 complete transection also had consistently reduced (<6 g) 50% tactile withdrawal threshold (**Fig. 3B**). To confirm that both spontaneous and evoked measurements were associated with true pain related behaviors, we performed "place escape avoidance preference" (PEAP) testing and confirmed that withdrawal of the forepaws from the von Frey stimulus represented active pain aversion to the stimulus. Rats with T3 complete transection spent significantly more time in the light chamber when the dark chamber was paired with stimulation by a 6g von Frey filament. In contrast, sham rats spent equal time in the light and dark chamber even when paired with stimulation in the dark chamber (**Fig. 3C**). A single dose of gabapentin acutely alleviated established tactile allodynia for 3 hours in rats with T3 complete transection (**Fig. 3D**). Additionally, the proximity of the injury to the cervical spinal cord and forelimb sensory circuitry was likely related to development of tactile allodynia; reduced forepaw withdrawal threshold was not apparent in rats after T11 complete transection (**Fig. 3E**).

Our preliminary data indicated that severe SCI rats exhibited cold allodynia. During this cycle we confirmed and expanded our studies. We tested the forepaws with a probe held at room temperature and at 13°C. Sham control and naive rats maintained a consistent withdrawal frequency (approximately 50%) over the duration of the study (**Fig. 4A, C**). Rats with T3 severe compression and T3 complete transection consistently exhibited increased forepaw withdrawal in response to contact with the 13°C cold probe, suggesting enhanced sensitivity to cold stimuli (**Fig. 4A, C**). Paw withdrawal frequency in response to the room temperature probe was comparable across groups at all time points (**Fig.4B, D**), indicating that the elevated withdrawal frequency was in response to temperature rather than contact with the probe. To determine whether injury impaired homeostatic heat regulation, as has been reported in patients with chronic SCI (Khan et al., 2007) (this could have impacted perception of cold stimuli), core and forepaw temperature was evaluated in awake rats with T3 transection, the more severe lesion, thirteen weeks after injury. No differences were identified between rats with T3 complete transection, sham surgery or naïve groups (**Fig. 4E**). We conclude that severe SCI is associated with at-level tactile and cold allodynia and spontaneous pain.

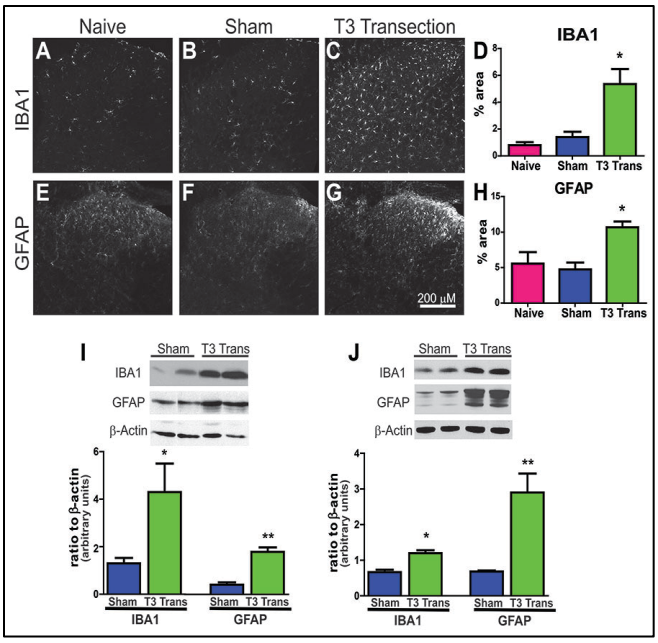


Fig. 5. Increased IBA1 and GFAP expression in C6-C8 spinal dorsal horn after severe SCI. Immunofluorescence microscopy was performed to detect IBA1 in ***A.*** Naïve ***B.*** Sham control and ***C.*** T3 complete transected rats 4 weeks after injury***. D.*** Pooled quantification of C6-C8 IBA1 immunofluorescence intensity. GFAP expression in ***E.*** Naïve ***F.*** Sham control and ***G.*** T3 complete transected rats 4 weeks after injury***. H.*** Pooled quantification of C6-C8 CGRP immunofluorescence intensity. Quantification of immunofluorescence intensity is expressed as mean ± SEM. n=3-5/group. *p<0.01 compared to naïve sham control by one-way ANOVA. Scale bar= 200 µM. Iba1 and GFAP levels were determined by immunoblot analysis in extracts of rat C6-C8 cervical dorsal spinal cord ***I.*** 4 weeks and ***J.*** 8 weeks after T3 complete transection. Blots were re-probed for β-actin as a loading control. Two representative rats from each group are shown. Equal amounts of cellular protein (20 µg) were loaded into each lane and subjected to SDS-PAGE. N=4/group. *p<0.05, **p<0.01 by t-test compared to sham control. Data are expressed as mean ± SEM.

Specific Objective: Identify molecular and cellular mechanisms by immunolabeling and immunobotting in spinal cord segments and DRGs (includes early and late timepoints). We identified key cellular and molecular mediators in key pain processing centers including the DRG and spinal dorsal horn. In our original grant application we showed preliminary data indicating that spinal glia

have increased immunoreactivity for GFAP, a marker of astrocytes and Iba1, a marker of microglia. We have now replicated and confirmed these findings at both early and later timepoints, 4 and 8 weeks. Glial activation in the spinal dorsal horn is a hallmark of neuropathic pain (Watkins et al., 2006) and has been associated with the development of SCI pain in moderate contusion injuries in rodent models (Carlton et al, 2009). Immunoreactivity of the microglial marker IBA1 was significantly increased throughout the dorsal horn in animals with T3 complete transection compared to naïve and sham controls (**Fig. 5A-D**) 4 weeks post-injury ($p<0.05$; **Fig. 5D**). Immunoblotting of C6-C8 dorsal spinal cord tissue both 4 and 8 weeks after T3 complete transection further confirmed significant elevations in IBA1. Rats with SCI had 4–fold greater levels of IBA1 compared to sham controls 4 weeks after injury ($p<0.05$, **Fig. 5I**) and persistent 1.6-fold elevations compared to sham animals at 8 weeks post-injury ($p<0.05$, **Fig. 5J**). We also observed significantly increased GFAP immunolabeling in C6-8 spinal dorsal horn 4 weeks after complete T3 transection ($p<0.05$ compared to naïve and sham controls; Fig. **5E-H**). Immunoblotting for GFAP 4 and 8 weeks following T3 complete transection confirmed these findings: GFAP was increased 4-fold after 4 weeks of injury ($p<0.05$, **Fig. 5I**), and further increased to a 6-fold difference from sham control after 8 weeks of injury ($p<0.05$, Fig. **5J**).

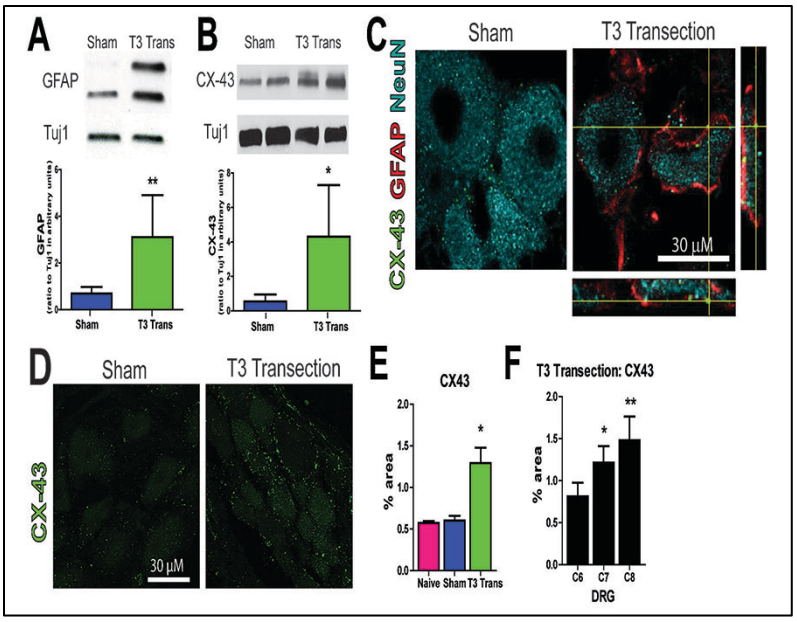


Figure 6. **SGCs become active in DRGs several segments rostral to injury site**. ***A***. GFAP levels and ***B.*** Connexin-43 were determined by immunoblot analysis in extracts of rat C6-C8 DRGs 4 weeks after injury. Blots were re-probed for β-neuronal tubulin (Tuji) as a loading control. Equal amounts of cellular protein (10 μg) were loaded into each lane and subjected to SDS-PAGE. Quantification of immunoblot results is expressed as mean ± SEM. N=3-9/group. *p<0.05, **p<0.01 by t-test compared to sham control. Dual labeling immunofluorescence microscopy was performed to detect ***C.*** Connexin-43 (green), GFAP (red) and NeuN (cyan) expression in a C7 DRG from a sham and T3 transected rat, 4 weeks after injury. Connexin-43 is primarily expressed in the perineuronal region surrounding NeuN, and co-localizes with GFAP following complete transection. ***D.*** Maximum projection z-stack of triple labeling immunofluorescence including connexin-43, GFAP and NeuN expression in the C7 DRG collected from naïve, sham and T3 complete transected rats. ***E.*** Pooled quantification of the percent area of connexin-43 immunoreactivity in C6-C8 DRG. **F.** Quantification of the percent area of connexin-43 immunoreactivity separated into C6-C8 DRGs collected from rats 4 weeks following T3 complete transection. Data are expressed as mean ± SEM. n=3-5/group. *p<0.05, **p<0.01 compared to C6 by one-way ANOVA followed by Tukey's *post-hoc* test.

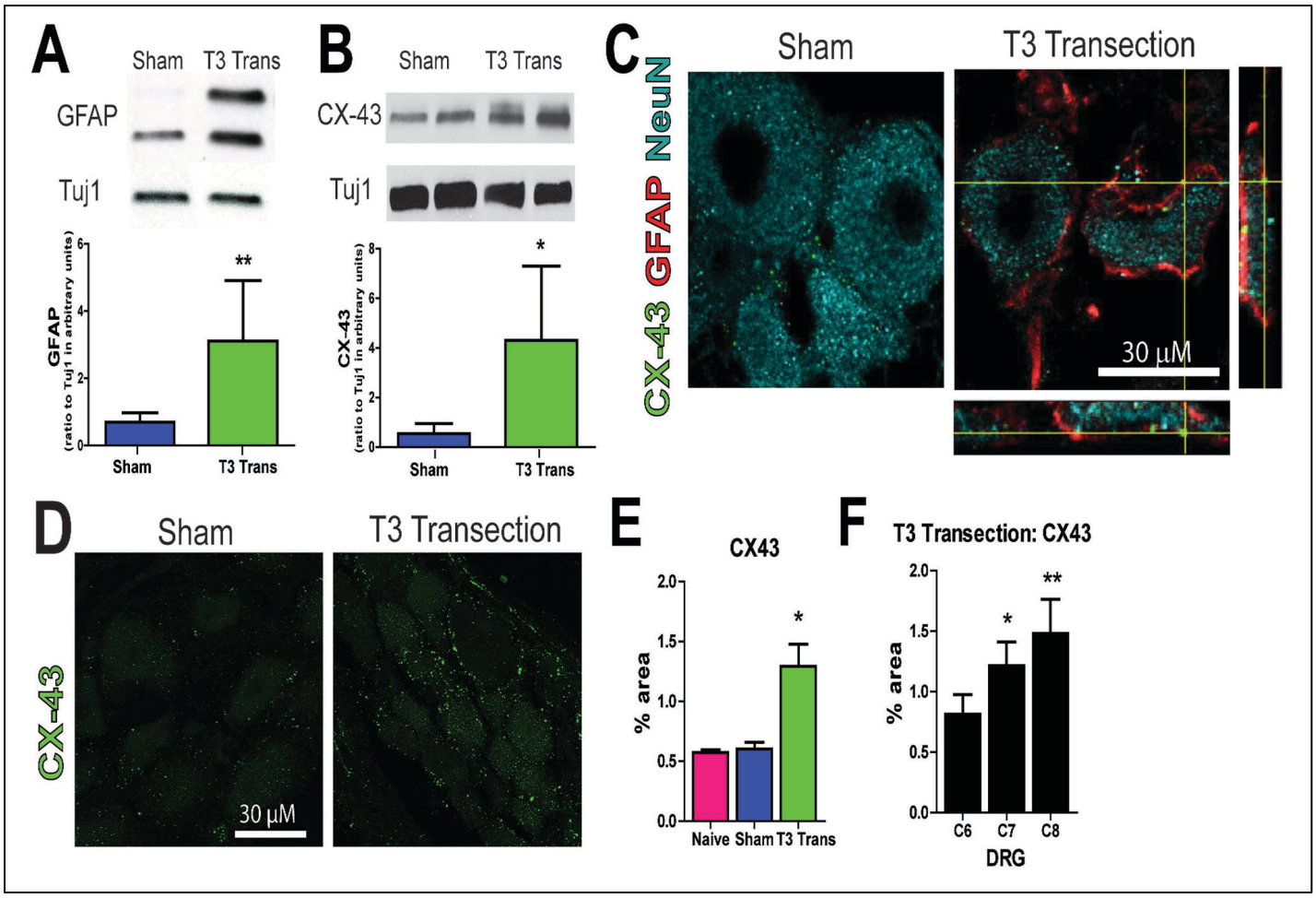


Fig. 7. SGCs become active in DRGs several segments rostral to injury site. ***A***. GFAP levels and ***B.*** Connexin-43 were determined by immunoblot analysis in extracts of rat C6-C8 DRGs 4 weeks after injury. Blots were re-probed for β-neuronal tubulin (Tuji) as a loading control. Two representative rat DRGs are shown. Equal amounts of cellular protein (10 μg) were loaded into each lane and subjected to SDS-PAGE. Quantification of immunoblot results is expressed as mean ± SEM. N=3-9/group. *p<0.05, **p<0.01 by t-test compared to sham control. Dual labeling immunofluorescence microscopy was performed to detect ***C.*** Connexin-43 (green), GFAP (red) and NeuN (cyan) expression in a C7 DRG from a sham and T3 transected rat, 4 weeks after injury. Connexin-43 is primarily expressed in the perineuronal region surrounding NeuN, and co-localizes with GFAP following complete transection. ***D.*** Maximum projection z-stack of triple labeling immunofluorescence including connexin-43, GFAP and NeuN expression in the C7 DRG collected from naïve, sham and T3 complete transected rats. ***E.*** Pooled quantification of the percent area of connexin-43 immunoreactivity in C6-C8 DRG. **F.** Quantification of the percent area of connexin-43 immunoreactivity separated into C6-C8 DRGs collected from rats 4 weeks following T3 complete transection. Data are expressed as mean ± SEM. n=3-5/group. *p<0.05,

We are also interested in whether chronic SCI pain is associated with ongoing peripheral sensitization since this was reported both above and below the level of injury following moderate SCI (Yang et al.,

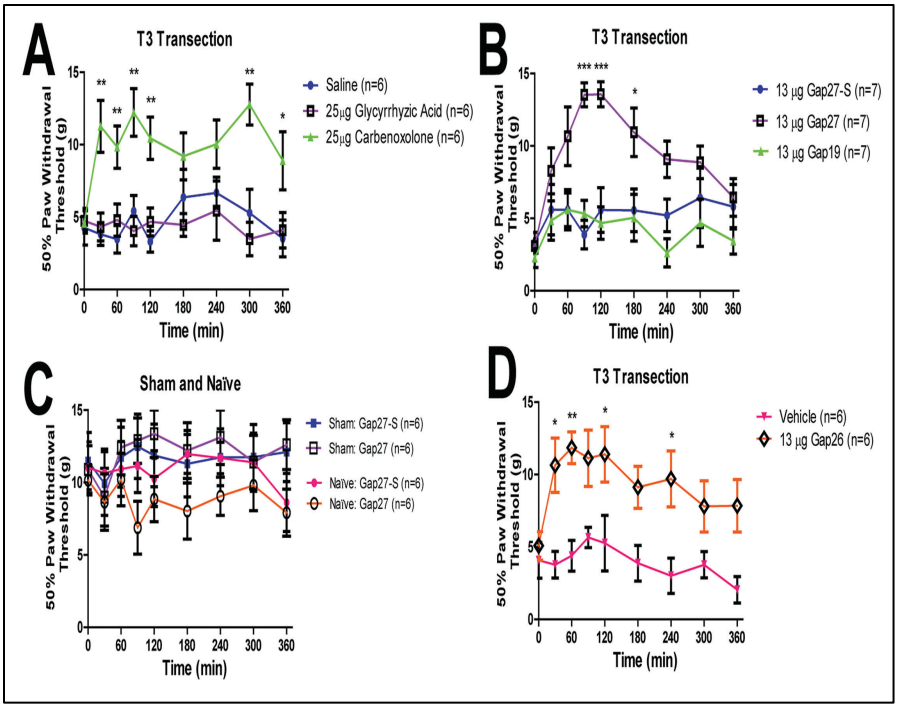


Fig. 8. Blocking connexin-43 alleviates established allodynia after T3 complete transection ***A.*** Effects of carbenoxolone (25 µg IT) or glycyrrhyzic acid (25 µg IT) on tactile withdrawal threshold 4 weeks after T3 complete transection. ***B.*** Effects of Gap27-scramble, Gap27 or Gap19 (13 µg IT) on forepaw tactile withdrawal threshold 4 weeks after T3 complete transection. ***C.*** Effects of Gap27 (13 µg IT) on forepaw withdrawal threshold in naïve or sham control rats. ***D.*** Effects of Gap26 (13 µg IT) or vehicle control on forepaw withdrawal threshold 4 weeks after complete transection. Forepaw tactile withdrawal threshold is expressed as mean ± SEM. N=6-7/treatment group. *p<0.05, **p<0.01 compared to saline treatment by two-way ANOVA followed by Bonferroni *post-hoc* test.

2014; Carlton et al., 2009; Bedi et al., 2010). GFAP levels also reflect satellite glial cell (SGC) activation in the DRG that sensitize primary sensory neurons following peripheral injury-induced neuropathic pain states (Fenzi et al., 2001). We evaluated GFAP expression in the C6-C8 dorsal root ganglia (DRG) to determine whether SGCs several segments rostral to the injury site are activated after severe T3 injury. Indeed, GFAP expression was significantly upregulated in DRGs in rats 4 weeks following T3 complete transection compared to uninjured controls (P<0.05; **Fig. 6A**). Connexin-43 is involved in coupling between adjacent SGCs, and its upregulation following peripheral injury has been associated with the development of neuropathic pain (Ohara et al., 2008). T3 complete transection significantly upregulated connexin-43 expression in C6-C8 DRGs 4 weeks following SCI (P<0.05 compared to sham control; **Fig. 6B**). Triple labeling immunofluorescence of C7 DRGs confirmed an increase of GFAP labeling in SGCs after injury that co-localized with connexin-43 (**Fig. 6C**). Quantification of connexin-43 immunolabeling in the C6-C8 DRG confirmed that connexin-43 expression was increased 4 weeks after injury (P<0.05 compared to naïve and sham control; **Fig. 6D-E**). Furthermore, the quantity of connexin-43 immunolabeling was dependent on proximity of the DRG to the lesion site, with significantly greater connexin-43 expression observed in the C8 and C7 DRGs from injured rats relative to C6 (**Fig. 6F**). Taken together, these data indicate that molecular

and cellular changes occur in DRG neurons rostral to the injury site following severe SCI that may contribute to the development of chronic pain states.

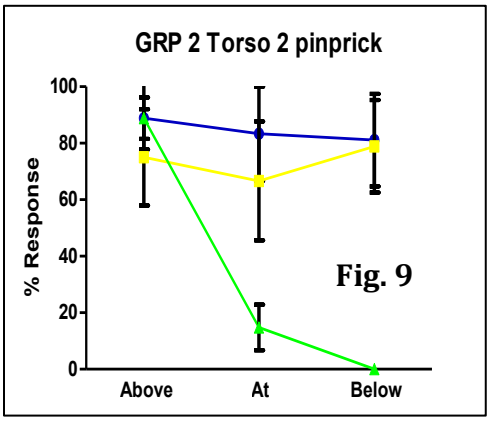


Fig. 9

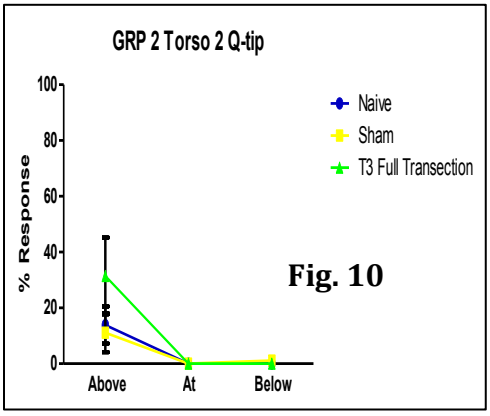


Fig. 10

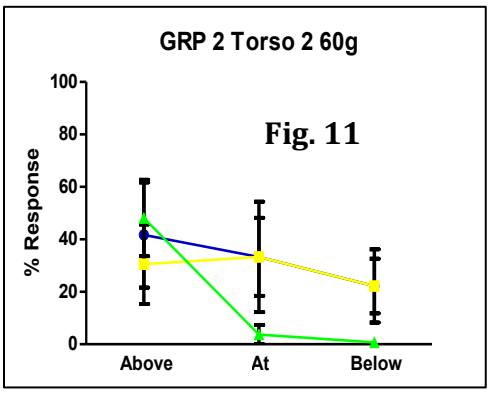
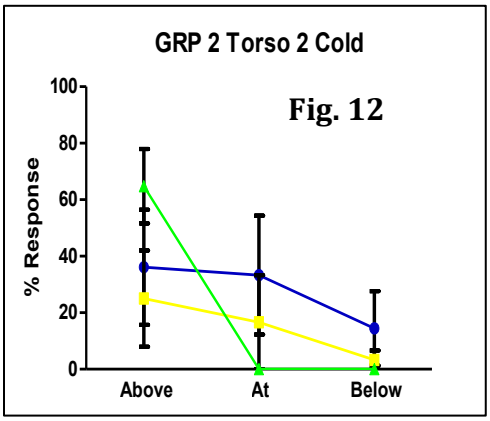


Fig. 11

Treatment with Connexin-43 Blockers Significantly Reduces Severe Post-SCI Pain. The function of connexin-43 in pain states (Chen et al., 2014) and our observation of increased connexin-43 in both the C6-C8 spinal cord and corresponding DRGs following severe SCI raised the possibility that this gap junction may be operational in the maintenance of severe SCI pain. To test this hypothesis, rats with severe SCI and established pain related behaviors (4 weeks) were treated with carbenoxolone, which disrupts gap junctions, but is also reported to have off-target effects, including blocking voltage-gated Ca^{++} channels, P2X7 and NMDA receptors (Juszczak et al., 2009) and glycyrrhizic acid, which is structurally similar to carbenoxolone and therefore shares its off-target effects, without inhibiting gap junctions (Juszczak et al., 2009). Carbenoxolone (25 µg, IT) significantly increased paw withdrawal threshold within 1 hour of administration, whereas glycyrrhyzic acid (25 µg, IT) and saline control had no effect (**Fig. 8A**). Next, selective peptide blockers of connexin-43 were used (Chen et al., 2014). Administration of Gap27 (13 µg, IT), which blocks both connexin-43 and -37 significantly elevated paw withdrawal threshold within 90 minutes of administration, whereas the Gap27 scramble control (Gap27-S, 13 µg, IT) had no effect (**Fig. 8B**). We also tested the effect of Gap19 (13 µg, IT), which selectively blocks connexin-43 hemichannels (Abudara et al., 2014) and found no effect on paw withdrawal threshold (**Fig. 8B**). The efficacy of Gap27 was not due to an effect on normal reflex sensitivity as it had no effect of paw withdrawal threshold in naïve and control subjects (**Fig. 8C**), suggesting that the efficacy in T3 transected rats is due to the pathological contribution of connexin-43 to reduced paw withdrawal threshold. Lastly, Gap26 (13 µg, IT), which selectively blocks



connexin-43 gap junctions and hemichannels significantly elevated paw withdrawal threshold within 30 minutes of administration relative to vehicle control (**Fig. 8D**). Collectively, these data suggest that blocking connexin-43-mediated communication between cells alleviates established tactile allodynia in the forepaws 4 weeks after T3 complete transection. These studies (**Fig. 1-8**) are published this year in *Experimental Neurology.*

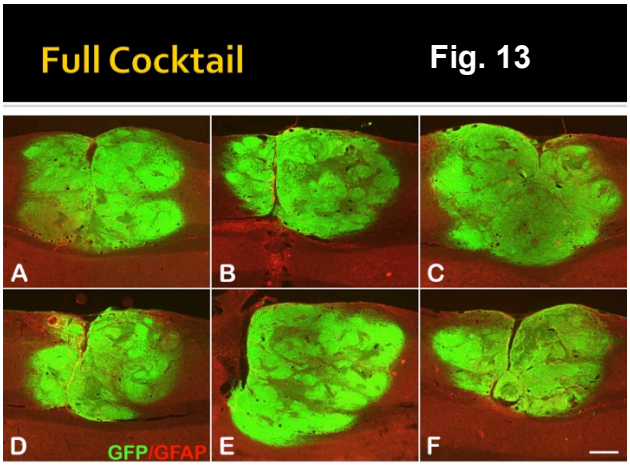
Specific Objective: Examine Effects of Neural Progenitor Cell Grafts on Pain Related-Behaviors and Molecular Mechanisms after Severe SCI. These activities includes work proposed in the Major Task 2 (Subtasks 1) of the Statement of Work (SOW). As outlined in the SOW and timeline, we have just initiated studies performed T3 complete transection in F344 rats with grafted neural progenitor cells into the lesion site. This quarterly report includes work proposed in the Major Task 1 (Subtask 3) and Major Task 2 (Subtask 2) of the Statement of Work (SOW). As outlined in the SOW, we performed T3 complete transection. Naïve and sham animals served as controls. A new pain testing modality was initiated: torso testing. outgrowth from the grafted material.

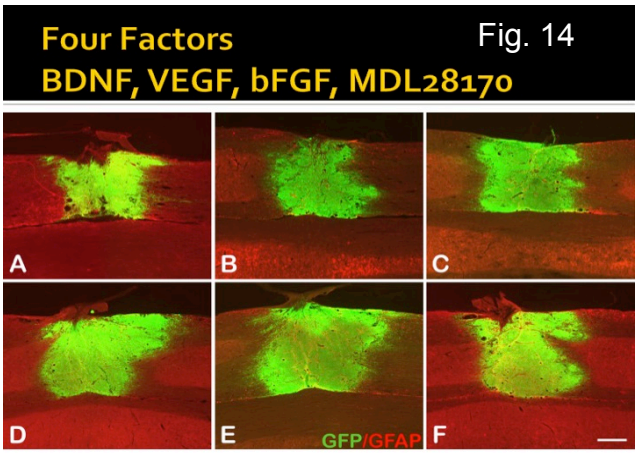
Torso testing after T3 complete transection: a model of severe SCI.

Torso testing determines whether a centralized pain response is elicited *above*, *below* and *at* the level of the injury at the appropriate dermatome. We tested several types of stimuli (Q-tip handle, 60g VF filament, 16G needle prick and 4C probe) and recorded responses in pilot studies. The back of rat was shaved and a grid of dots was drawn on each rat to indicate stimulus points (pts). We included 6 pts above level, 3 pts at level and 18 pts below level (**Fig. 9**). Stimuli were presented for 5 seconds all within the same session with 5-minute intervals between. All responses were recorded, and data are expressed as % response. Rats were tested 13 weeks post T3 complete lesion, a timepoint when pain is established in other stimulus evoked modalities (tactile allodynia). Groups included naïve (n=6), sham (n=6) and T3 full transection (n=9). **Fig. 10** shows the % response of rats stimulated with a Q-tip. T3 full transection showed the greatest response above level, but no response in at or below level. Naive and sham groups had no response to Q-tip stimulus (**Fig. 10**), most likely due to the low intensity of the stimuli. Next, we evaluated a 60 g von Frey filament. Both sham and naive

groups responded whereas, the T3 transected group only responded above the lesion site (**Fig. 11**). The pinprick group shows the highest response rate, as anticipated in both the sham and naïve animals. The T3 transected group responded most robustly above the lesion site and interestingly, at the same % response compared to the other groups (**Fig. 12**). Some response was noted at level. All groups responded to cold stimuli that were less apparent below the injury site. The T3 transected group only responded above the lesion site.

Optimization of NSC grafting with growth factor cocktails- We also began our first set of grafting studies using E14 rat neural stem cells (NSCs) into the lesion sites after T3 complete transection. We report viable cells in the lesion site up to 13 weeks. To determine whether we could enhance NSC survival and axon To determine whether grafting conditions in the T3 lesion site were optimized, we grafted E14 rat NSCs into lesion sites incubated with *full cocktail*, as previously described (Lu et al., 2012) or in a *four factor cocktail*. The four factor cocktail represents a more defined cocktail that is likely more suitable for clinical translation. Three million NSCs expressing GFP were grafted into the lesion site in both treatment groups. Preliminary results in **Fig. 13** and **14** demonstrate that both the full (n=6) and four (n=6) factor cocktail are capable of promoting NSC survival. However, the actual number of grafted cells appears to be greater in the full cocktail compared with the four factors cocktail. Whether this is due to proliferation of NSCs or increased NSC viability remains to be determined. Greater axon extension was observed with the full cocktail. The full cocktail had no effect on tactile allodynia (**Fig. 15**). These data indicate that NSC grafting requires further optimization with various growth factor combinations for improved axon extension.





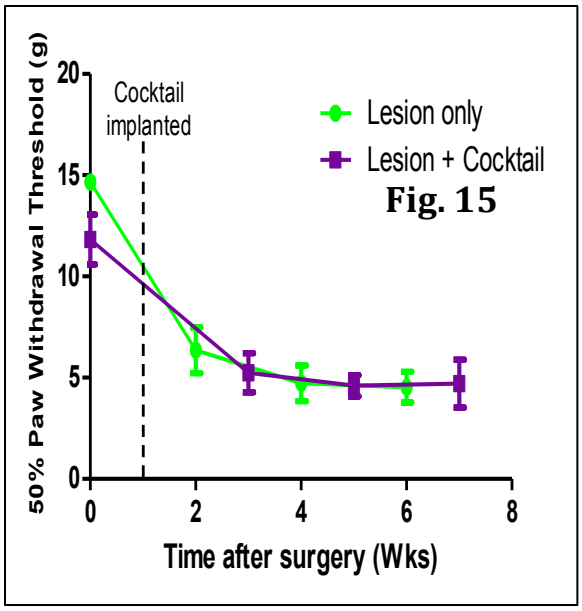


Fig. 15

Identification of Extracellular Matrix in the Lesion site before and after grafting E14 rat NSCS

Some of the grafted neural stem cells did not extend long axons due to "rift-like" formations in the lesion sites and thus, optimization studies were needed prior to evaluating pain outcomes. We examined the surrounding extracellular matrix (ECM) composition of the lesion site to determine whether this environment was modulated by NSC grafting. The extracellular matrix proteins present in the lesion site after T3 complete transection are unknown. We designed studies to identify which ECM proteins were expressed in the lesion site (after 2 weeks) prior to grafting NSC cells. Subsequently, we determined whether grafting NSC cells changed the ECM environment after 14 weeks. We examined collagen IV and laminin as these ECM proteins are essential for functional axon growth (Ide, 2006). GFAP, an astrocyte marker, was also used to determine the extent of astrocyte proliferation/gliosis. We report that collagen IV and lamina were abundantly present in the lesion site and co-localized on occasion with GFAP (). In addition, the lesion site was surrounded by GFAP expression, indicating a presence of astroglial scar formation. However, GFAP expression was largely excluded within the scar. To test the hypothesis that grafting NSC into the lesion cavity alters the ECM environment, rats with T3 transection were grafted with GFP expressing NSCs (3 million cells in full cocktail) 2 weeks after injury and immunolabeled for ECM markers 13 weeks post grafting. **Fig. 17** shows that grafted cells form a "rift" that includes the expression of both laminin and collagen IV within the rift and grafted borders. These results indicate that the types of ECM proteins present in the lesion site do not change as a result of the NSC grafting. A permissive environment for axon growth that include laminin and collagen IV, remain present. However, "rift" like structures were present that may interfere with continuous axon

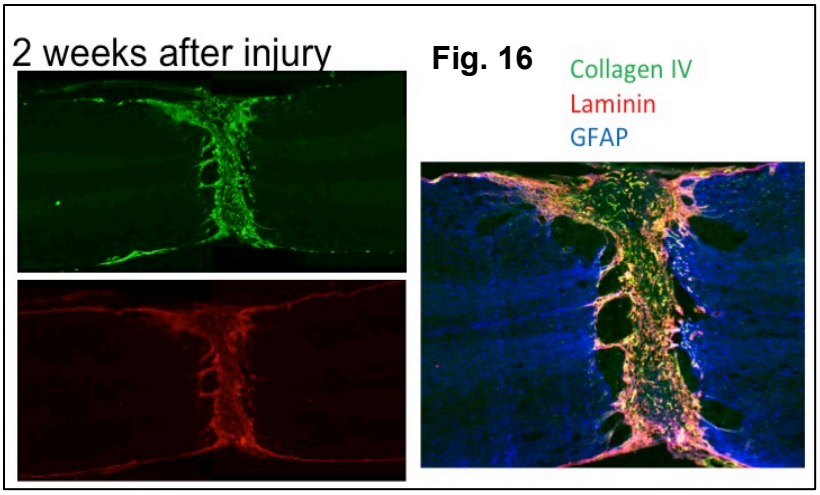


Fig. 16

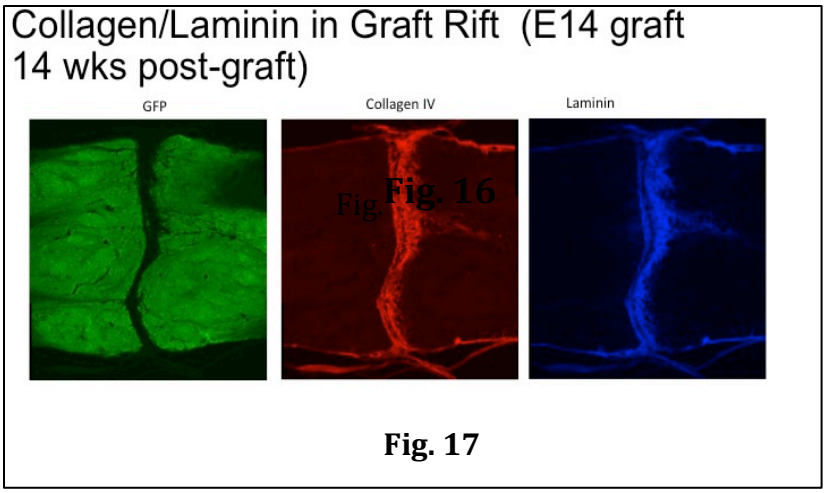


Fig. 17

growth.

Inhibition of cell proliferation at the lesion site by administration of AraC - To continue optimizing grafting of NSC into the T3 transected lesion site, we performed studies with AraC, a potent inhibitor of cell proliferation. Rats were infused with 2% AraC supplied by a subcutaneous osmopump for one week post T3 transection. E14 spinal cord-derived NSCs expressing GFP were grafted into closed lesion cavities in 11-factor cocktail. Rats were sacrificed 5 weeks after grafting. Spinal cord sections were collected and immunolabled with collagen IV and laminin. **Fig. 18** shows preliminary results that AraC treatment did not 1) eliminate the rift; 2) alter the permissive environment of ECM proteins; and 3) affect NSC survival.

Because grafted NSCs survive and extend axons in full cockatail, we proceeded with proposed SCI pain studies using the T3 transection model. T3 complete transection resulted in extensive loss of hindlimb function that was associated with a score <3 on the 21 point BBB locomotor scale (**Fig. 19, BBB Scores**). NSC grafted group (E14 cells) did not improve scores and is in contrast to what was reported by Lu *et al.*, 2012. Deficits remained severe in both groups and did not differ substantially over 13 weeks. At 13 weeks core temperature was unchanged in all groups (**Fig. 20; Core Temp**). Next we determined whether spontaneous pain was present in rats that have sustained severe SCI. We evaluated spontaneous paw lifting in the forelimbs. Forelimb measures represent responses from neural circuitry located at least two spinal segments above the lesion, and could result from plastic rearrangements evoked by the

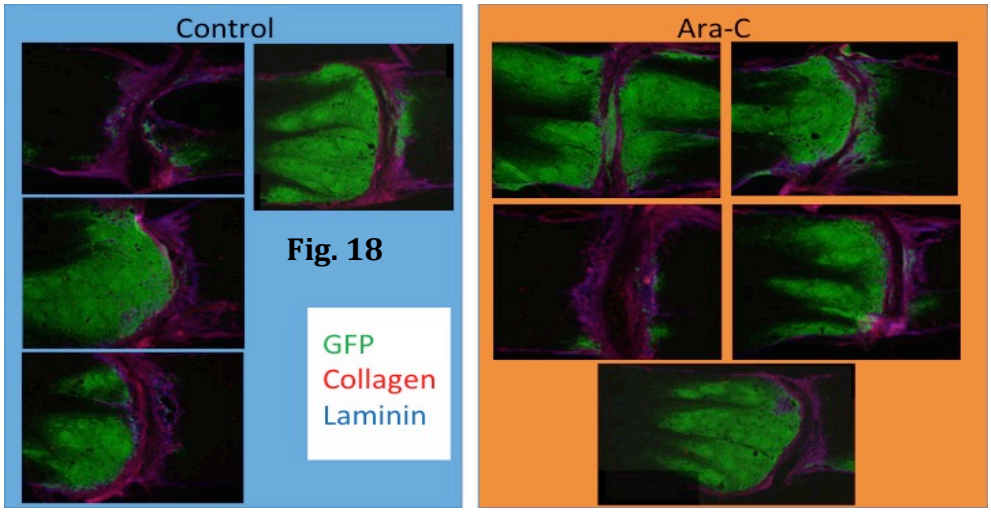
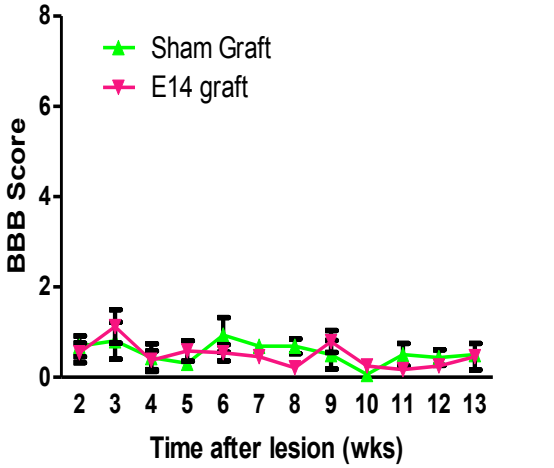


Fig. 18



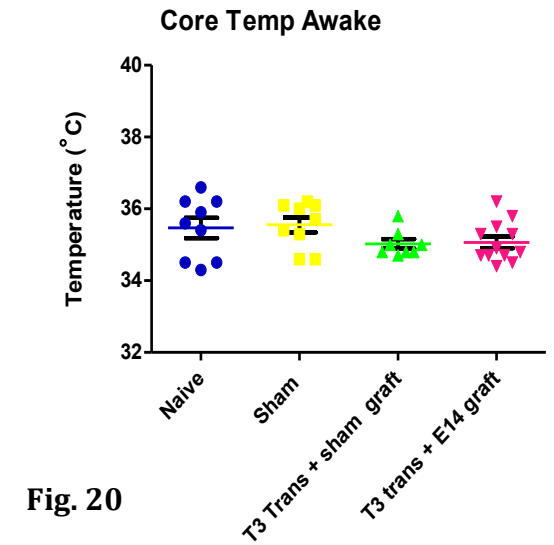


Fig. 20

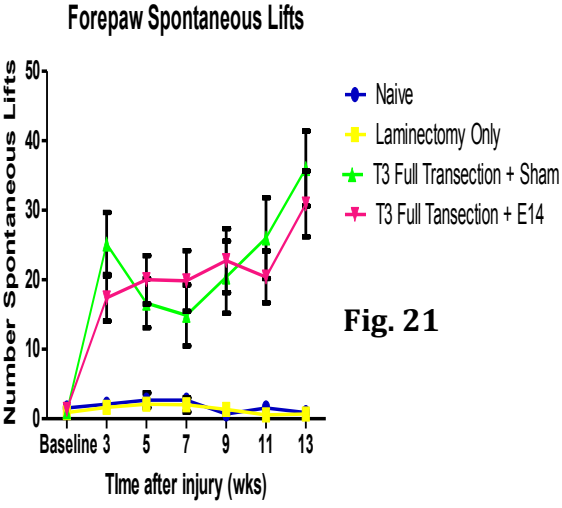


Fig. 21

proximity to the lesion site. In naïve and laminectomy only rats (controls), spontaneous forepaw lifting behavior was negligible (fewer than 3 lifts/4 min) and remained unchanged over the duration of the study (**Fig. 21**). In contrast, rats with either T3 severe transections or T3 complete transection with grafted NSCs exhibited significant spontaneous forepaw lifting over 12-13 weeks compared to controls. There was no difference between the lesioned and lesioned with grafted NSCs (**Fig. 21**). We are in the process of compiling evoked pain related behavior data (tactile allodynia, cold allodynia) and determining whether the axons extended formed functional neuronal relays.

What opportunities for training and professional development has the project provided?

- The project includes a post doctoral fellow, Dr. Corinne Lee-Kubli, PhD. Dr. Lee-Kubli is an expert in testing pain related behaviors in rodents. Over the last year, she has been trained in surgeries that include, T3 complete transection and T3 compression under the direction of Dr. Tuszynski. This training is now complete. Her primary mentor is myself, Dr. Campana. I have assisted Dr. Lee-Kubli in refining her pain behavior testing techniques, culture of neural stem cells, immunoblotting and immunofluorescense with one-on-one training . Dr. Lee-Kubli has presented her work at the Asilomar Conference, Carmel CA and at our Department of Anesthesiology Research Retreat, San Diego CA. These interactions, mentorship, one-on-one trainings and presentations have facilitated Dr. Lee-Kubli's professional career.

How were the results disseminated to communities of interest?

"Nothing to Report"

What do you plan to do during the next reporting period to accomplish the goals?

We are on schedule with the SOW. Our plan is to continue to optimize the NSC grafting and evaluate pain related behaviors (spontaneous, evoked and operant) in our established severe SCI models. We will confirm that graft derived axons extend and integrate into host tissues by GFP immunofluorscence. We will confirm that axons make neural relays by immunolabeling with synaptophysin. Pain related behaviors will be tested and include spontaneous forepaw lifting, tactile and cold allodynia, PEAP and torso testing.

IMPACT: What was the impact on the development of the principal discipline(s) of the project?

We describe a model of T3 severe SCI that consistently results in forelimb pain-related behaviors that are comparable to reports of pain experienced by patients with SCI. Gliopathy is associated with the development and maintenance of the chronic pain state, including peripheral alterations in connexin-43 and central alterations in astrocytes, microglia and neurotransmitters. Notably, therapeutic targeting of the glial-neuronal interface significantly ameliorated SCI-related pain.

- **What was the impact on technology transfer?**

  "Nothing to Report."

- **What was the impact on society beyond science and technology?**

  Our recently published manuscript improves the knowledge base for scientist studying neuropathic pain and spinal cord injury. In particular, we characterize the cellular and molecular mechanisms of severe SCI associated with pain states.

CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

  “Nothing to Report”

PRODUCTS: Publications, conference papers, and presentations

- **Journal publications.**

Lee-Kubli CA, Ingves M, Henry KW, Shiao R, Collyer E, Tuszynski MH, Campana, WM (2016) Analysis of the behavioral, cellular and molecular characteristics of pain in a severe rodent spinal cord injury. *Experimental Neurology* 278:91-104.

- **Other publications, conference papers, and presentations.**

Lee-Kubli CA, Ingves M, Henry KW, Shiao R, Collyer E, Tuszynski MH, Campana, WM (2015) Mechanisms associated with persistnet pain states after severe rodent spinal cord injury. ISNR Asilomar, Carmel, CA.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Primary Investigator (PI): Wendy Campana, PhD (no change)

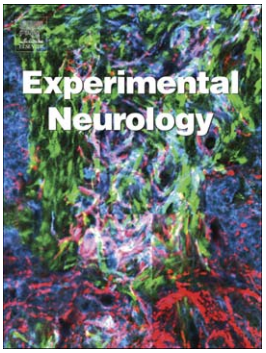
- Mark Tuszynski, MD, PhD (no change)
- Corinne Lee-Kubli, PhD (no change)
- Ken Henry, PhD (no change)

SPECIAL REPORTING REQUIREMENTS

Not applicable

APPENDICES: Please see attachment.

DO NOT RENUMBER PAGES IN THE APPENDICES.



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Analysis of the Behavioral, Cellular and Molecular Characteristics of Pain in Severe Rodent Spinal Cord Injury

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Running head: Spinal Cord Injury Pain

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Number of words in Introduction: 558

Number of words in Discussion: 2035

Abstract

Human SCI is frequently associated with chronic pain that is severe and refractory to medical therapy. Most rodent models used to assess pain outcomes in SCI apply moderate injuries to lower thoracic spinal levels, whereas the majority of human lesions are severe in degree and occur at cervical or upper thoracic levels. To better model and understand mechanisms associated with chronic pain after SCI, we subjected adult rats to T3 severe compression or complete transection lesions, and examined pain-related behaviors for three months. Within one week after injury, rats developed consistent forepaw pain-related behaviors including increased spontaneous lifts, tactile allodynia and cold sensitivity that persisted for three months. Place escape avoidance testing confirmed that withdrawal of the forepaws from a von Frey stimulus represented active pain-related aversion. Spontaneous and evoked pain-related measures were attenuated by gabapentin, further indicating that these behaviors reflect development of pain. Spinal level of injury was relevant: rats with T11 severe SCI did not exhibit forepaw pain-related behaviors. Immunoblotting and immunofluorescence of C6-C8 spinal dorsal horn, reflecting sensory innervation of the forepaw, revealed: 1) expansion of CGRP immunoreactivity in lamina I/II; 2) increased GAP-43 expression; and 3) increased IBA1, GFAP and connexin-43 expression. These findings indicate that aberrant pain fiber sprouting and gliopathy occur after severe SCI. Notably, satellite glial cells (SGCs) in C6-C8 DRGs exhibited increases in GFAP and connexin-43, suggesting ongoing peripheral sensitization. Carbenoxolone, a gap junction inhibitor, and specific peptide inhibitors of connexin-43, ameliorated established tactile allodynia after severe SCI. Collectively, severe T3 SCI successfully models persistent pain states and could constitute a useful model system for examining candidate translational pain therapies after SCI.

Introduction

Pain develops in approximately two-thirds of patients with spinal cord injury (SCI) [24;54;55]. Chronic neuropathic pain is the most debilitating type of post-SCI pain, and in the majority of patients manifests as spontaneous ongoing pain with accompanying mechanical and cold allodynia [4]. Mechanisms underlying chronic post-SCI pain are poorly understood and thus, drug development for treatment has been limited and remains a high priority.

The majority of human SCI are severe, characterized by complete loss of sensory and motor function below the lesion, and occur most commonly at cervical or high thoracic spinal level [50]. Yet, the majority of pre-clinical models of SCI pain have focused on excitotoxic lesions, ischemic injuries or incomplete contusion injuries of the low thoracic spinal cord [59]. Contusion injury models are widely considered to be the most mechanistically relevant to human SCI; however, when administered in a manner that results in injury of moderate severity, this model can spare white matter at the lesion site and allows for substantial recovery of locomotor function. In contrast, most human SCI is severe in degree with little, if any, recovery of neurological function. Moreover, it is difficult to assess the extent of sensory functional sparing below an incomplete rodent SCI lesion [19,38,57] and to determine whether responses to sensory testing reflect pain-related behaviors rather than alterations in reflex responses, including spasticity [5]. One approach to circumvent these problems has been to evaluate symptoms of “at-level” pain in regions that are not denervated by the SCI. Common traumatic injury models include evaluating torso allodynia after moderate thoracic contusion [34] and contralateral forelimb allodynia after cervical hemicontusion [19]. Nevertheless, the percent of rats that develop signs of allodynia in these models remains low, perhaps due to variability in tissue sparing, meaning that a much greater number of rats needs to be used to develop a mechanistic understanding of neuropathic pain following SCI.

Although some molecular mechanisms associated with enhanced evoked withdrawal or chronic pain behaviors after SCI have been identified [5,7,8,12,16,28-31,35] relatively little is known about mechanisms underlying chronic post-SCI pain

behaviors in the forelimbs, particularly following injuries in the cervical or upper thoracic spinal cord [19]. Because spinal cord levels (C7/C8) mediating forelimb sensation in rats are anatomically adjacent (within 3-5mm) to cervical or upper thoracic spinal cord injuries, pain outcomes in the forelimbs likely reflect “at-level” pain, a clinically relevant type of pain experience for SCI patients [24]. Furthermore, there are few studies of “at- and above-level” pain behaviors and outcomes in rodent models of the most common form of human injury, severe injury [41].

In this study, we report that forelimb pain-related behaviors consistently develop and persist following severe T3 SCI over a 3-month period. Forelimb pain is associated with aberrant sprouting of CGRP immunoreactive axons, but without alterations in the IB4-binding population or GAD67 immunoreactivity at C6-C8 spinal levels. Glial activation is also observed. Notably, connexin-43 and GFAP are increased in C6-C8 DRGs after severe SCI, suggesting ongoing peripheral sensitization. Forepaw tactile allodynia is ameliorated by treatment with the gap junction decoupler, carbenoxolone, and connexin-43 peptide inhibitors, highlighting the importance of glial intercommunication in chronic pain associated with severe SCI. Our results suggest that T3 SCI successfully models persistent pain states after SCI, particularly “at-level” pain experienced by patients. Severe SCI constitutes a useful model system for examining effects of candidate translational pain therapies.

Methods

Experimental Design

Studies were performed using a total of 102 adult female Fischer 344 rats (150-160 g; Harlan Industries, San Diego, CA, USA). We chose female rats for these studies because their bladders can be more readily emptied following severe SCI compared to male rats, resulting in greater subject survival. It has been reported that pain prevalence rates and descriptions of pain do not differ between male and female patients with SCI [17,62] and there is little evidence to suggest that female rats are less suitable for studies of pain due to the variability introduced by associated with the estrus cycle [47]. In previous studies, pain-related behaviors developed in both male and female rats after SCI; accordingly, we used female rats.

Experimental subjects underwent either sham surgery (N=30), T3 complete transection (N=30) or severe T3 compression (N=12). Comparisons were made to animals that underwent T11 transections (N=9). We used T3 level lesions because this is the highest spinal level at which a severe spinal cord lesion in the rat is consistent with subject survival; severe lesions located more rostral than T3 result in persistent forelimb dysfunction and rats are subsequently unable to move about their enclosures to access nutrition and fluid. Beginning one week after T3 severe lesions, subjects underwent biweekly assessment of spontaneous paw lifts, withdrawal thresholds using von Frey hairs and cold withdrawal threshold. Studies were performed for 12-13 weeks. Two animals were excluded at study completion; one was excluded due to an incomplete lesion (BBB score >8) and the other due to death at week 3. In additional cohorts of rats, C6-C8 DRGs and spinal dorsal horn tissues were collected 4 and 8 weeks after injury for immunoblotting and immunofluorescence (T3 transected, N=13; sham, N=11; naïve, N=8).

Animal Care

Animals were housed 2-3 per cage with free access to food and water in a vivarium approved by the American Association for the Accreditation of Laboratory Animal Care. All animal studies were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the VA hospital, San Diego, CA and following the IASP Guidelines for Use of Animals in Research.

Spinal cord injury and sham surgeries

After two weeks of acclimation and collection of baseline behavioral values, rats underwent surgery under deep anesthesia using a combination of ketamine (75 mg/kg), xylazine (2.6 mg/ml), and acepromazine (0.5 mg/ml). An incision was made in the skin of the back over the T2 spinous process. After clearing of the muscle, the dorsal aspect of the T3 vertebra was removed. For severe compression, the cord was compressed for 5 seconds using 1.5 mm-wide mosquito forceps locked and completely closed (Fig. 1). This injury procedure is a modification of the clip-compression model [47]. For

complete transection, a longitudinal incision was made in the dura with a #11 blade, leaving the surface of the cord exposed. Small iridectomy scissors were used to bilaterally transect the cord at two sites in the center of the T3 spinal segment located 1mm apart, and the 1mm segment between cuts was removed by aspiration. The muscles overlying the spinal cord were sutured and the skin incision was closed with surgical staples. Sham surgeries consisted of T3 laminectomy only. T11 complete transection was performed in the same way as T3 complete transection, only with laminectomy of the T9 vertebra, corresponding to the T11 spinal cord segment. Following surgery, rats were maintained in cages kept on heat pads (37 ° C) for 1 week and received banamine (1 mg/kg) and ampicillin (0.2 mg/kg) in Ringer's lactate for three days. Bladder care was performed twice daily at 12 hour intervals for the first two weeks following surgery, and thereafter once daily until rats could urinate on their own, approximately four weeks after surgery. Bladders were always expressed prior to acclimation for behavioral testing. Rats were given amoxicillin their drinking water over the duration of the experiment to prevent bladder infections that could confound behavioral results.

Locomotor assessment

BBB scoring was conducted weekly beginning 1-2 weeks after the initial injury, when animals were sufficiently recovered from surgery to enable scoring. Scoring was conducted following the established protocol of BBB [6]. Movements that occurred during bladder or bowel movement were excluded. Two experienced observers conducted testing.

Evaluation of pain-related behaviors

Testing of pain-related behaviors was conducted starting 1-3 weeks following injury, to allow rats to recover from the surgical procedures. All experiments were conducted in a

blinded fashion when possible (i.e. for drug administration studies and comparison of lesion levels) although the identities of lesioned vs. unlesioned animals were obvious.

Spontaneous forepaw lifts were determined while rats were contained in plexiglass chambers on a wire mesh grid. After 15 minutes of acclimation, spontaneous lifting of both forepaws was evaluated over a 4-minute period. This behavior frequently occurred in bursts of multiple alternating L-R paw lifts. Foot lifting associated with exploratory behavior, locomotion, body repositioning, and grooming was excluded [18].

Tactile withdrawal threshold was determined by testing rats in plexiglass chambers placed on a smooth stainless steel grid platform, allowing access from underneath the rat. The 50% paw withdrawal threshold to a series of calibrated von Frey filaments, (Kom Kare, Middletown, OH, USA) was assessed in the center of both hindpaws and forepaws before surgery (baseline) and at regular intervals following SCI using the up-down method [12] using a set of filaments calibrated to exert a force of 0.2–15g. Forepaw measurements were taken from the center of the forepaw while it was placed flat on the grid. Values from each pair of forepaws were averaged per time point.

Sensitivity to cold was measured using a Peltier device (TEC1-12706) that was attached to an aluminum heat sink and equipped with a 4 mm diameter aluminum rod [27]. Depending on the current, this device generates a consistent temperature ranging from 13°- 4° C at the tip of the rod. With rats placed on a metal grid within a plexiglass container, the cooled Peltier device was placed in contact with the center of each fore or hindpaw for 5 seconds or until a withdrawal was observed. Forepaw testing was conducted with the device cooled to 13 °C because naïve rats responded maximally at 10 °C. Testing was repeated 5 times for each paw, alternating between paws, and percent paw withdrawal was calculated for each pair of forepaws. At least 1 minute was allowed to pass before testing another paw in the same animal, and at least 5 minutes before testing the same paw. Withdrawal frequency was recorded in response to contact with the room temperature (RT) probe to control for the possibility that rats were reacting to the touch, rather than the temperature of the device. The probe was monitored periodically to verify that it was held at the expected temperature. Forepaw

and core temperatures were collected at the conclusion of the 13-week T3 complete transection study to exclude the possibility that changes in reactivity to temperature were due to differences in thermoregulation between groups.

Place-escape avoidance paradigm

The place-escape avoidance paradigm, modified from [39], was used to evaluate active pain related aversion to forepaw stimulation with a von Frey filament 4 weeks after SCI. Animals were evaluated in a test box composed of two (25 x 30 x 30) chambers separated by a central holding chamber (12 x 30 x 30) with smooth stainless steel grid flooring throughout. The light chamber had clear plexiglass walls and no ceiling, while the dark chamber had opaque walls and ceiling. The animals were placed in the central holding chamber and allowed to habituate for 5 min after which the separating walls were removed and rats were allowed to move freely throughout the box. Upon entry into the dark chamber, rats in the von Frey (VF) group were stimulated with a 6 g von Frey filament, corresponding to a force one step greater than the mean 50% withdrawal threshold of the group, once every 10 seconds, alternating between forepaws. The no stimulation (NS) group received no stimulation while in the dark chamber. In both groups, no stimulation was applied once rats crossed into the light side. Each animal was tested for 20 min, and the amount of time spent in the light side was recorded over 5 min time intervals. Time spent in the light side between 5-20 min was used as an indication of escape avoidance learning. Data were normalized to their corresponding NS group.

Evaluation of pharmacological effects

The effects of gabapentin (Tocris Bioscience, Bristol, United Kingdom) on spontaneous forepaw lifts in rats with a T3 complete transection (N=8) were studied over two days during the 13th week following SCI. The effect of gabapentin on forepaw tactile withdrawal threshold was evaluated 4 weeks after T3 complete transection (N=6). Rats were randomly assigned to the saline or gabapentin groups. Pain-related behaviors were observed at baseline and for 5 hours following intraperitoneal

administration of gabapentin (30 mg/kg i.p. dissolved in saline) or saline control. On the second day, after verifying that gabapentin effects had washed out, rats were assigned the opposite groups and the same experimental procedures were followed.

For evaluating the contribution of connexin-43 to maintenance of pain following severe SCI, drugs or vehicle were injected in a 5 µl volume, directly to the cervical spinal cord via an indwelling intrathecal (IT) catheter implanted 5 days prior to drug delivery. Catheters were constructed using an 15 mm long piece of polyethylene PE8 tubing (0.008 inch inner diameter, 0.014 inch outer diameter) fused with heat to a 5 cm long piece of PE10 tubing. A small amount of dental acrylic was used to help maintain the catheter at the base of the skull. The catheters were implanted as described elsewhere [57]. Briefly, rats were maintained under isoflurane anesthesia while the head was immobilized in a stereotaxic frame and an incision approximately 1 cm in length was made along the midline of the skull. A small incision was made in the underlying muscles perpendicularly to the midline of the skull, and the muscles were then blunt dissected to expose the atlanto-occipital membrane overlying the cisterna magna. A small nick was made in the membrane and the PE8 end of the catheter was gently inserted into the subarachnoid space. A small amount of saline was injected to verify that the catheter retained patency, before heat-sealing the PE10 end. Rats displaying forelimb locomotor deficits following implantation were excluded from the study. Drugs were administered in 10 µl volume following by 5 µl of saline to flush the catheter. The effects of carbenoxolone (25 µg, IT, dissolved in saline, Sigma-Aldrich) and glycyrrhizic acid (25 µg, IT, dissolved in saline, Sigma-Aldrich) on tactile allodynia were tested in 16 rats 4 weeks following T3 complete transection. The effects of Gap19 (13 µg, IT, dissolved in dI H_2O, Tocris Bioscience), Gap26, Gap27 or Gap 27-scramble (13 µg, IT, dissolved in dI H_2O, Anaspec) were tested in 21 rats 4 weeks following T3 complete transection. Rats were randomly assigned to treatments groups, and the experimenter remained blinded. Tactile withdrawal threshold was evaluated at baseline and over 5 hours following drug administration. Correct catheter placement was confirmed by laminectomy at the conclusion of the study.

Immunoblotting

Rats were anesthetized with isoflurane and decapitated. C6-C8 spinal dorsal horn and DRGs were prepared in RIPA buffer (PBS with 1% triton-X100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail and sodium orthovanadate). The protein concentration in tissue extracts was determined by bicinchoninic acid assay. An equivalent amount of cellular protein (20 µg per lane) was subjected to 4-20% gradient SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-HCl buffered saline, pH 7.4 with Tween 20 and incubated with the primary antibodies: GAD67 (1:5000,Millipore, MAB5406), GFAP (1:400, Chemicon, MAB360), Iba1 (1:2400, Thermo Scientific, PA5-27436) β-actin (1:10,000, Sigma-Aldrich, A2228), Tuj1 (1:1000, Covance, MMS-435P), Connexin-43 (1:400, Life Technologies, 71-0700) according to the manufacturer's recommendations. The membranes were washed and treated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoblots were developed using enhanced chemiluminescence (Amersham). Blots were scanned (Cannoscan) and densitometry was performed with Image J (NIH).

Immunofluorescence

Tissue used for histology was collected from deeply anesthetized rats that were transcardially perfused with 0.9% saline followed by 4% PFA. DRGs and spinal column were removed and kept in 4% PFA overnight at 4 °C and transferred to 30% sucrose prior to dissection and sectioning. For evaluation of the spinal lesion site, 30 µM horizontal tissue sections were collected every 6 sections and subsequently directly mounted onto slides and Nissl stained. Spinal cord cross-sections for IF were free floated. DRG sections were cut at 10 µM thickness. Sections were incubated overnight at 4 °C with biotin-conjugated IB4 (1:1000, Sigma L2140) or primary antibodies against: CGRP (1:1000, EMD Millipore ab15360), Iba1 (1:1000; Wako Chemicals 019-19741), GFAP (for spinal cord 1:1000; EMD Millipore mab360; for DRG 1:500, Dako Z0334), NeuN (1:1000, EMD Millipore MAB377), GAD67 (1:1000, EMD Millipore MAB5406), connexin-43 (CX-43; 1:500, Everest Biotech EB09301). Appropriate Alexa-Fluor 488,

594 or 647 secondary antibodies raised in donkey or goat (Life Technologies) were applied for 1 hr at room temperature. In control studies, the primary antibody was omitted. Nuclei were labeled with DAPI (Invitrogen, Eugene, OR). Slides are visualized using an Olympus BX 53 microscope. Pictures were taken with a mounted camera (Q Imaging Retina 2000R) and CellSens Digital Imaging computer software. Images were taken using consistent exposure times across all sections. A light level image was taken for identification of the dorsal horn. Images used for evaluation of CX-43 expression and co-localization were collected with an Olympus confocal microscope, (Fluoview FV1000-IX81) at 0.57 µM intervals using a 60x objective lens.

Immunofluorescence was quantified by evaluating six sections sampled throughout the C6-C8 region using ImageJ software (NIH). For evaluation of mean light intensity a region of interest was drawn around the periphery of the immunolabeled region of the dorsal horn and the fluorescent intensity within the region of interest was normalized to the mean from each naïve group. Percent area of Iba1 and GFAP was evaluated by measuring the number of pixels containing immunoreactivity above minimum threshold intensity, determined using the thresholding function of ImageJ, within the delineated dorsal horn. Connexin-43 intensity in the DRG was evaluated by taking a maximum projection Z-stack of images collected with the confocal microscope from C6, C7 and C8 DRG and calculating % area of the image that was occupied by connexin-43 immunoreactivity. Data collected from C6-C8 spinal cord and DRG were pooled together for each experimental subject. All quantification was performed in a blinded fashion.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism (v 5.0; La Jolla, CA, USA) using either unpaired one- or two-tailed t-test, paired two-tailed t-test, one-way ANOVA followed by Tukey's or Dunnett's *post-hoc* tests for multiple comparisons, or two-way repeated measures ANOVA followed by Bonferroni's *post-hoc* test, as indicated. Data are reported as group mean ± SEM.

Results

Functional Impairment Following T3 Transection or Compression is Severe SCI

Both T3 severe compression and T3 complete transection resulted in extensive loss of hindlimb function that was associated with a score <3 on the 21 point BBB locomotor scale (Fig. 1A,B). In rats with T3 severe compression, BBB scores rose slightly beginning 7 weeks following injury (Fig. 1A). Nonetheless, deficits in both groups remained severe and did not differ substantially. T3 severe compression lesion histology demonstrated a band of disrupted parenchyma across the compression site with partial surrounding cavitation (Fig. 1C). T3 complete transection injuries were characterized by a narrow lesion surrounded by inflammatory cell infiltrates (Fig. 1D). Lesion histology confirmed the absence of spared tissue in the lesion site of all rats included in the study.

Severe SCI Induces Spontaneous Forelimb Pain-Related Behaviors

Spontaneous pain is a prominent manifestation of at-level pain reported by SCI patients [4]. To determine whether spontaneous pain was present in rats that have sustained severe SCI, we evaluated spontaneous paw lifting in the forelimbs. Forelimb measures represent responses from neural circuitry located at least two spinal segments above the lesion, and could result from plastic rearrangements evoked by the proximity to the lesion site. In control rats, spontaneous forepaw lifting behavior was negligible (fewer than 3 lifts/4 min) and remained unchanged over the duration of the study (Fig. 2A,B). In contrast, rats with either T3 severe compression (Fig. 2A) or T3 complete transection (Fig. 2B) exhibited significant spontaneous forepaw lifting over 12-13 weeks compared to controls (repeated measures ANOVA, $p<0.01$, comparing T3 lesioned groups to their respective control groups). In rats with T3 severe compression, spontaneous forepaw lifting declined 8 weeks after injury ($p<0.05$ comparing Wk 8, 10 and 12 to Wk 2 by one-way repeated measures ANOVA followed by Tukey's test), whereas these behaviors increased over time in rats with T3 complete transection. The reduction in spontaneous forepaw lifting was not linked to variability of locomotor outcome as spontaneous lifting

behavior in the three rats with BBB scores greater than 3 were not significantly different from those of the four rats with BBB scores less than 3 (by unpaired two-tailed t-test comparing average weeks 8-12 spontaneous forepaw lifts and linear regression).

T3 complete transection rats were treated with gabapentin (30 mg/kg, i.p.) following 13 weeks of injury. Gabapentin is a drug that alleviates pain in a subset of human patients with SCI [42]. Gabapentin significantly reduced spontaneous forepaw lifting behavior, with a peak efficacy 60 to 120 min after administration (Fig. 2C). This suggests that spontaneous forepaw lifting may be a pain-related behavior, rather than being secondary to other aspects of severe thoracic injury such as postural changes.

Next, we determined whether vertebral level of injury influenced spontaneous forepaw lifting behavior. Rats that underwent a T3 complete transection exhibited significantly increased spontaneous forepaw lifting when assessed six weeks after injury (overall ANOVA, $p<0.01$; *post-hoc* Tukey's $p<0.001$ comparing T3 transection to control), whereas rats that underwent T11 complete transection did not display this behavior (Fig. 2D). These findings suggest that spontaneous forepaw lifting behaviors are a manifestation of at-level pain as proximity of the injury to the cervical spinal cord is necessary for their development.

Severe SCI Induces Evoked Forelimb Pain-Related Behaviors

At-level pain sensations in human patients of SCI also comprise abnormal evoked responses such as allodynia in response to light touch and hypersensitivity to cold stimuli [4]. We examined both tactile and cold forelimb allodynia in rats with severe SCI. Sham control and naïve rats had a 50% forepaw withdrawal threshold that consistently remained in the non-allodynic range (>10g) over the duration of the study (Figs. 3A,B). However, rats with T3 severe compression exhibited consistently reduced 50% tactile withdrawal threshold beginning 1 week post-injury and continuing through the end of the study 12 weeks later (Fig. 3A). Every rat had an average 50% paw withdrawal value less than 6.5 g over the 1-12 week period, indicating consistent development of allodynia. Rats with T3 complete transection also had consistently reduced (<6 g) 50%

tactile withdrawal threshold (Fig. 3B). Place escape avoidance preference (PEAP) testing confirmed that withdrawal of the forepaws from the von Frey stimulus represented active pain aversion to the stimulus. Rats with T3 complete transection spent significantly more time in the light chamber when the dark chamber was paired with stimulation by a 6g von Frey filament. In contrast, sham rats spent equal time in the light chamber even when the dark chamber was paired with stimulation (Fig. 3C). A single dose of gabapentin acutely alleviated established tactile allodynia for 3 hours in rats with T3 complete transection (Fig. 3D). Additionally, the proximity of the injury to the cervical spinal cord and forelimb sensory circuitry was likely related to development of tactile allodynia; reduced forepaw withdrawal threshold, indicating allodynia, was not apparent in rats after T11 complete transection (Fig. 3E).

Next, we evaluated cold allodynia in severe SCI rats. We tested the forepaws with a probe held at room temperature and at 13°C. Sham control and naive rats maintained a consistent withdrawal frequency (approximately 50%) over the duration of the study (Fig. 4A, C). Rats with T3 severe compression and T3 complete transection consistently exhibited increased forepaw withdrawal in response to contact with the 13°C cold probe, suggesting enhanced sensitivity to cold stimuli (Fig. 4A, C). Paw withdrawal frequency in response to the room temperature probe was comparable across groups at all time points (Fig.4B, D), indicating that the elevated withdrawal frequency was in response to temperature rather than contact with the probe.

To determine whether injury impaired homeostatic heat regulation, as has been reported in patients with chronic SCI [37] (this could have impacted perception of cold stimuli), core and forepaw temperature was evaluated in awake rats with T3 transection, the more severe lesion, thirteen weeks after injury. No differences were identified between rats with T3 complete transection, sham surgery or naïve groups (Fig. 4E).

Neurotransmitter and Glial Mechanisms Associated with At-Level SCI Pain

In an effort to identify cellular and molecular mechanisms that are associated with severe SCI pain-related behaviors in forelimbs, we examined known mediators of

pain processing in the cervical spinal dorsal horn mediating forelimb sensation and movement (C7-C8). Rats with T3 transection exhibited significantly increased CGRP immunoreactivity ($p<0.05$ comparing lesioned to naïve and sham animals; Fig. 5A-D), but not increased binding of IB4 (Fig. 5E-H) in laminae I-II of spinal dorsal gray matter 4 weeks after injury. Further, there was significantly ($p<0.05$) more overlap between the regions innervated by CGRP and the IB4-binding population, respectively (Fig.5I-L), suggesting sprouting of pain fibers in the dorsal spinal laminae. We also observed a significant increase in the growth cone marker GAP-43 in the C6-C8 spinal dorsal horn, 8 weeks after T3 complete transection ($p<0.05$ compared to sham control; Fig.5M). GAD67 levels were not significantly different in C6-C8 spinal dorsal horn after severe SCI (sham=3.02$\pm$0.22 *vs.* Injury=3.1$\pm$0.18 arbitrary units at 8 weeks).

Glial activation in the spinal dorsal horn is a hallmark of neuropathic pain [54] and has been associated with the development of SCI pain in moderate contusion injuries in rodent models [11]. Immunoreactivity of the microglial marker IBA1 was significantly increased throughout the dorsal horn in animals with T3 complete transection compared to naïve and sham controls (Fig. 6A-D) 4 weeks post-injury ($p<0.05$; Fig. 6D). Immunoblotting of C6-C8 dorsal spinal cord tissue both 4 and 8 weeks after T3 complete transection further confirmed significant elevations in IBA1. Rats with SCI had 4–fold greater levels of IBA1 compared to sham controls 4 weeks after injury ($p<0.05$, Fig. 6I) and persistent 1.6-fold elevations compared to sham animals at 8 weeks post-injury ($p<0.05$, Fig. 6J). We also observed significantly increased GFAP immunolabeling in C6-8 spinal dorsal horn 4 weeks after complete T3 transection ($p<0.05$ compared to naïve and sham controls; Fig. 6E-H). Immunoblotting for GFAP 4 and 8 weeks following T3 complete transection confirmed these findings: GFAP was increased 4-fold after 4 weeks of injury ($p<0.05$, Fig. 6I), and further increased to a 6-fold difference from sham control after 8 weeks of injury ($p<0.05$, Fig. 6J).

The observation of increased GFAP expression led us to evaluate expression of connexin-43, the primary gap junction protein expressed by astrocytes [48,59]. Connexin-43 was significantly upregulated 4 and 8 weeks following T3 complete transection in immunoblot lysates from C6-C8 dorsal spinal cord ($p<0.05$ compared to

sham control, Fig. 7A-C). We performed double labeling to identify the cells types within the spinal dorsal horn that express connexin-43 after injury. Connexin-43 co-localized with GFAP (Fig. 7D), confirming primary expression by astrocytes, but not NeuN (Fig. 7E). However, connexin-43 immunoreactivity was also found in close apposition to IBA1 positive structures, suggesting that it could also be present on microglia within the dorsal horn (Fig. 7F), consistent with observations following brain stab injuries [22].

Changes in DRG Satellite Glial Cells after Severe SCI: Upregulation of GFAP and Connexin-43

Peripheral sensitization has been reported both above and below the level of injury following moderate SCI [7; 11; 58]. GFAP levels reflect satellite glial cell (SGC) activation in the DRG that sensitize primary sensory neurons following peripheral injury-induced neuropathic pain states [24]. We evaluated GFAP expression in the C6-C8 dorsal root ganglia (DRG) to determine whether SGCs several segments rostral to the injury site are activated after severe T3 injury. Indeed, GFAP expression was significantly upregulated in DRGs in rats 4 weeks following T3 complete transection compared to uninjured controls ($P<0.05$; Fig. 8A).

Connexin-43 is involved in coupling between adjacent SGCs, and its upregulation following peripheral injury has been associated with the development of neuropathic pain [46]. T3 complete transection significantly upregulated connexin-43 expression in C6-C8 DRGs 4 weeks following SCI ($P<0.05$ compared to sham control; Fig. 8B). Triple labeling immunofluorescence of C7 DRGs confirmed an increase of GFAP labeling in SGCs after injury that co-localized with connexin-43 (Fig. 8C). Quantification of connexin-43 immunolabeling in the C6-C8 DRG confirmed that connexin-43 expression was increased 4 weeks after injury ($P<0.05$ compared to naïve and sham control; Fig. 8D-E). Furthermore, the quantity of connexin-43 immunolabeling was dependent on proximity of the DRG to the lesion site, with significantly greater connexin-43 expression observed in the C8 and C7 DRGs from injured rats relative to C6 (Fig. 8F). Taken together, these data indicate that molecular and cellular changes

occur in DRG neurons rostral to the injury site following severe SCI that may contribute to the development of chronic pain states.

Treatment with Connexin-43 Blockers Significantly Reduces Severe Post-SCI Pain

The function of connexin-43 in pain states [14] and our observation of increased connexin-43 in both the C6-C8 spinal cord and corresponding DRGs following severe SCI raised the possibility that this gap junction may be operational in the maintenance of severe SCI pain. To test this hypothesis, rats with severe SCI and established pain related behaviors (4 weeks) were treated with carbenoxolone, which disrupts gap junctions, but is also reported to have off-target effects, including blocking voltage-gated Ca^{++} channels, P2X7 and NMDA receptors [36] and glycyrrhizic acid, which is structurally similar to carbenoxolone and therefore shares its off-target effects, without inhibiting gap junctions [36]. Carbenoxolone (25 µg, IT) significantly increased paw withdrawal threshold within 1 hour of administration, whereas glycyrrhyzic acid (25 µg, IT) and saline control had no effect (Fig. 9A). Next, selective peptide blockers of connexin-43 were used [13]. Administration of Gap27 (13 µg, IT), which blocks both connexin-43 and -37 [13], significantly elevated paw withdrawal threshold within 90 minutes of administration, whereas the Gap27 scramble control (Gap27-S, 13 µg, IT) had no effect (Fig. 9B). We also tested the effect of Gap19 (13 µg, IT), which selectively blocks connexin-43 hemichannels [2], and found no effect on paw withdrawal threshold (Fig. 9B). The efficacy of Gap27 was not due to an effect on normal reflex sensitivity as it had no effect of paw withdrawal threshold in naïve and control subjects (Fig. 9C), suggesting that the efficacy in T3 transected rats is due to the pathological contribution of connexin-43 to reduced paw withdrawal threshold. Lastly, Gap26 (13 µg, IT), which selectively blocks connexin-43 gap junctions and hemichannels significantly elevated paw withdrawal threshold within 30 minutes of administration relative to vehicle control (Fig. 9D). Collectively, these data suggest that blocking connexin-43-mediated communication between cells alleviates established tactile allodynia in the forepaws 4 weeks after T3 complete transection.

Discussion

Herein we demonstrate the development of consistent, significant and persistent "at-level" pain-related behaviors in two rat models of clinically relevant, severe SCI: T3 severe compression and T3 complete transection. Pain-related behaviors, including spontaneous paw lifts, tactile allodynia and cold allodynia, persisted for 3 months and are consistent with clinical reports of pain modalities that develop in patients with SCI. Motor outcome scores confirmed the ongoing severe nature of these injuries, establishing direct relevance to human injury, which is most commonly severe in extent (ASIA A)[45]. Nearly all preceding studies of pain in models of traumatic SCI have used moderate contusion models of injury that can be associated with considerable white matter sparing [11; 34]. Spared axonal projections from below the level of the lesion may contribute plastic changes that depart from mechanistic consequences that are likely after severe human injury. We propose that more clinically relevant, severe injuries merit greater exploration in translational SCI studies [52], including the study of SCI pain.

T3 complete transection and severe compression models result in major locomotor deficits and produce well-defined, consistent and clearly interpretable results [39]. As candidate regenerative therapies have now shown functional benefits in models of severe SCI [40], testing of pain outcomes in these severe models is becoming increasingly important for potential clinical translation. Preclinical models of SCI that will allow for simultaneous evaluation of functional efficacy and impact on clinically relevant neuropathic pain outcomes are needed. Furthermore, severe compression and transection lesions are consistent and reproducible such that all of the subjects in our studies exhibited pain-related behaviors across all tested modalities, in contrast to studies using moderate contusion injuries wherein only subsets of animals develop "at"- or "above-level" neuropathic pain [19; 32; 33; 44].

We placed injuries in the high thoracic spinal cord (T3) and detected changes in forelimb pain-related behaviors. This likely reflects "at-level" pain due to the proximity of the lesion site to forelimb spinal cord segments that process sensory inputs (C7-C8). Cervical and high thoracic injuries constitute the majority of human injuries and are often associated with "at-level" pain [50]. Notably, forelimb pain-related behaviors

developed in our rats with T3 but not T11 severe SCI. While previous studies have reported that lower thoracic (T10-T13) contusion injuries can result in forelimb pain [7; 11; 18; 33], this may be a feature of incomplete lesion models, as models of T9 and T13 complete transection did not develop forepaw allodynia [18; 41]. Development of "above-level" pain in human patients has been reported following SCI [26] but is now believed to occur only in cases in which patients have co-morbid conditions, such as diabetes mellitus, that predisposes them to development of neuropathic pain [55]. The current IASP classification of SCI pain, which no longer includes "above-level" pain, defines "at-level" pain as one that is detected within three dermatomes of the injury site [48]. It is likely that pain in the forelimbs of our rats after T3 severe lesions reflects "at-level" pain, because 3 spinal levels in humans (~30mm) exceed the 5-6 mm distance from T3 to C7-C8 spinal segments in rats. Likewise, evaluation of torso allodynia following T9 complete transection showed spread of sensitivity over a 6 cm^2 region above the level of injury [41], making it reasonable that "at-level" pain following T3 complete transection would encompass the dermatomes innervating the forepaws. We chose to focus our studies on the development of "above-level" in rodents pain due to clinical relevance; "at-level" pain is persistent, disruptive to quality of life and frequently rated categorized as the most disturbing pain experienced by patients with SCI [10; 23; 49].

Development of "at-level" pain in patients is frequently co-incident with or predisposes patients to development of "below-level" pain, so understanding these mechanisms may inform therapeutic options for "below-level" pain [59]. "Below-level" pain assessment in a complete injury model can be challenging to interpret because there are injury-related alterations in reflex responses to sensory stimuli; in the presence of a complete spinal cord transection, it is unlikely that these responses are consciously perceived [5]. However, in the present study we examined "at- and above" -level responses to pain stimuli, wherein supraspinal nociceptive projections are not interrupted. Moreover, previous studies have revealed no alterations in "above-level" simple reflex responses and have successfully modeled "at-level" pain using ischemic, excitotoxic and contusive lesions [19; 33; 56; 60]. Few studies have evaluated "at-level" pain in models of severe SCI [41]. Additionally, we confirmed that rats with T3 complete

transection were capable of perceiving forelimb stimuli as being aversively painful using the PEAP, which demonstrated that injured rats spent significantly more time in the light chamber when the dark chamber was paired with intermittent stimulation (6g von Frey filament). Therefore, the responses observed in the present study appear likely to represent pain responses.

We selected our sensory modalities on the basis of clinical reports of spontaneous pain, tactile allodynia and increased cold sensitivity in patients with “at-level” SCI pain [4]. Spontaneous pain is the most difficult to evaluate in rodents because of their obvious inability to report pain. Indeed, there are no ideal methods for measuring spontaneous pain. Thus, we evaluated ongoing spontaneous pain by evaluating forepaw lifting behavior in acclimated rats, as described by others [20]. Control rats do not spontaneously flinch their paws, but rats with both T3 complete transection and severe compression exhibited a stereotypic lifting behavior that was not associated with postural adjustment. This behavior may be comparable to spontaneous foot lifting behaviors after injury which correlate with spontaneous firing of primary afferents [20]. Furthermore, forepaw lifting was alleviated by gabapentin, a drug that partially alleviates neuropathic pain in humans [42] and rodents [8], suggesting that forepaw lifting behavior was indicative of spontaneous pain. However, gabapentin has also been reported to have effects on sedation [25] and spasticity [38], and could have reduced the number of spontaneous forepaw lifts by impacting these systems. Yet, development of spasticity is typically reported only in deafferented regions below the level of the injury [3], and the dose of gabapentin we used (30 mg/kg) is below the dose reported to yield sedation [25; 31].

Rats with severe T3 SCI also consistently developed forelimb tactile allodynia, which was reversed by gabapentin, and increased sensitivity to cold, both of which have been reported by patients with “at-level” pain [26]. “At-level” cold allodynia has previously been reported in a model of photochemically-induced ischemic injury [27], while conscious aversion to cold flooring has been reported in a rat model of spinal stenosis [53]. Acute “at-level” cold sensitivity to acetone has also been reported following T9 complete transection [41]. Here, we have shown that, not only does cold

allodynia manifest in the forepaws of rats with T3 severe lesions, it persists over at least a 12-13 week duration following injury. Taken together, our results suggest that severe T3 injury equally recapitulate signs of neuropathic pain that are experienced by humans who have sustained SCI, reflecting the relevance of the severe T3 lesion to the clinical condition.

The altered responses of both neurons and glia play key roles in ongoing neuropathic pain [20; 54]. Severe SCI induced an expansion of peptidergic axons (CGRP) in lamina I/II. No changes were observed in non-peptidergic afferents (IB4-binding population). Sprouting of new peptidergic branches into C6-C8 spinal dorsal horn was confirmed by expression of GAP43, a growth cone marker. These findings are similar to those reported after moderate injuries, reflecting expanded nociceptive inputs to dorsal horn neurons [17; 20; 54]. However, in contrast to SCI of moderate severity, we did not observe changes in spinal GABAergic tone (altered GAD67 expression), which is thought to be associated with a greater risk of developing neuropathic pain [21; 29; 43]. Thus, severe SCI is associated with mechanisms of neuronal plasticity that are both common and distinct from previous reports in models of incomplete injury. Increased expression of excitatory neurotransmission has been reported in SCI [15; 19].

Glial cells are important for maintaining neuro-architecture and physiological homeostasis in the spinal cord [54]. After SCI, spinal glia become activated, a feature that is considered to be one of the mechanistic underpinnings of neuropathic pain [29]. After severe T3 SCI, IBA1 and GFAP expression are significantly increased in cervical spinal segments; interestingly, IBA1 is increased to a greater extent at 4 weeks than 8 weeks. This may be due to rapid mobilization of microglia and macrophages that may limit the severity of parenchymal injury, although specific macrophage responses can also worsen injury [28]. In contrast, GFAP expression was significantly increased to a greater extent at 8 weeks after injury, as compared to 4 weeks. Our findings are consistent with models of neuropathic, inflammatory and post-operative pain, which report an increase in the number of activated microglia within a few days, followed by astrocyte activation. As microglial activation begins to attenuate, astrocyte activation

and hypersensitivity persists [30; 50; 51]. Chronic pain may be maintained, rather than initiated, by astrocyte activation, which has important implications for the development of potential therapeutics for SCI pain. Furthermore, targeting spinal astrocytes several segments rostral to the injury site may be beneficial in SCI.

We describe novel cellular responses in the DRG innervating the lower cervical segments, including increased expression of GFAP and connexin-43 in SGCs, following development of forepaw pain-related behaviors. Thus, severe SCI involves alterations in sensory processing pathways in the pain circuit that are located outside the spinal cord. Indeed, moderate contusion injuries lead to peripheral sensitization that can be linked to alterations in intrinsic properties of primary neurons [7; 11; 51; 58]. In this study, we identified a novel extrinsic mechanism within the DRG that may regulate peripheral sensitization: ongoing activation of SGCs and upregulation of connexin-43. Connexin-43 is a gap junction protein that is involved in ion transport and communication between adjacent SGCs [46] and can also contribute to release of neurotransmitters and cytokines through hemichannels that open into the extracellular space [9]. The contribution of connexin-43 in peripheral glial activation in DRGs has not been previously appreciated in the context of persistent SCI pain. However, increased connexin-43 expression in SGCs has been shown to contribute to the development of neuropathic pain states following peripheral nerve injury [35].

A potential role for connexin-43 in severe SCI pain is supported by our data demonstrating that IT administration of the gap junction blocker carbenoxolone directly to the C7-C8 spinal segments reduced tactile allodynia 4 weeks following SCI. Furthermore, IT delivery of Gap27, which blocks connexin-43 and -37, and Gap26, which selectively blocks connexin-43, robustly alleviated forepaw tactile allodynia as well. In contrast, Gap19, which selectively blocks connexin-43 hemichannels through which neurotransmitters could be released, had no effect, suggesting that elevated connexin-43 maintains pain-like behaviors primarily through enhanced coupling between adjacent cells. These drugs had no effect on forepaw sensitivity in naïve and sham rats. Though the primary site of drug delivery through an IT catheter is the spinal cord itself, intrathecally delivered agents also have access to the DRG [1], making it

likely that drug delivery would have targeted gap junctions in both the DRG and spinal cord. Others have shown that mice with genetic deletion of connexin-43 in astrocytes had attenuated hindlimb withdrawal to tactile and heat stimuli following moderate T10 SCI [14], although it should be noted that *cre* expression in this experiment was under the GFAP promoter and could also have impacted connexin-43 levels in peripheral SGCs. Therefore, the relative contribution of SGCs in the periphery or astrocytes in the spinal cord to ongoing severe SCI pain related behaviors requires further study. Nonetheless, connexin-43 blockade or downregulation may be a viable therapeutic target for both treatment of neuropathic pain and improving regeneration following SCI, as treatment with an anti-sense oligonucleotide against connexin-43 reduced tissue swelling and disruption and improved locomotor outcomes in a model of moderate compressive SCI [16].

We describe a model of T3 severe SCI that consistently results in forelimb pain-related behaviors that are comparable to reports of pain experienced by patients with SCI. Gliopathy is associated with the development and maintenance of the chronic pain state, including peripheral alterations in connexin-43 and central alterations in astrocytes, microglia and neurotransmitters. Notably, therapeutic targeting of the glial-neuronal interface significantly ameliorated SCI-related pain.

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Figure 1. Comparison of BBB scores in models of severe SCI. ***A.*** T3 severe compression (n=7). ***B.*** T3 complete transection (n=8). Data are expressed as mean ±SEM. ***C.*** Terminal lesion histology shows a band of disrupted parenchyma with associated cavitation in rats with T3 severe compression. ***D***. Rats with T3 compression showed a narrow lesion surrounded by inflammatory cell infiltrates. Horizontal sections through the lesion site are oriented rostral to caudal from left to right. Scale bar is 1mm.

Figure 2. T3 severe SCI induces forepaw spontaneous pain-related behaviors in rats with ***A.*** T3 severe compression compared to sham control. ***B***. T3 complete transection compared to naïve and sham control. Graphs show the number of forepaw lifts within 4 minutes over 13 weeks. N=6-9/group. *p<0.05, **p<0.01, ***p<0.001 compared to sham control and naive by two-way repeated measures ANOVA with Bonferroni *post-hoc* analysis. ***C.*** Effect of gabapentin (30 mg/kg i.p.) on spontaneous forepaw lifts when administered at 13 weeks after T3 complete transection. N=8/group. **p<0.01, ***p<0.001 compared to saline treatment by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* analysis. ***D***. Effect of spinal level of injury on spontaneous forepaw lifts 6 weeks after injury in sham control , T3 complete transection (T3 Trans) and T11 complete transection (T11 Trans). N=4/group. **p<0.01, ***p<0.001 by one-way ANOVA followed by Tukey's *post-hoc* test. Data are expressed as mean ± SEM.

Figure 3. Forepaw tactile allodynia develops after severe SCI. ***A.*** T3 severe compression compared to sham control. ***B.*** T3 complete transection compared to naïve and sham control. Forepaw tactile withdrawal thresholds were evaluated in rats over 13 weeks. N=6-9/group. *p<0.05, ***p<0.001 compared to sham control and naive by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* analysis. ***C.*** Place escape avoidance paradigm (PEAP) conducted 4 weeks after sham or T3 complete transection surgery. Data are expressed as "fold-difference" compared to non-stimulated (NS) N=5/group. *p<0.05 compared to NS by unpaired, two-tailed t-test. ***p<0.001 compared to sham by unpaired, two-tailed t-test ***D.*** Effect of gabapentin (30

mg/kg i.p.) on forepaw tactile withdrawal when administered at 4 weeks after T3 complete transection (n=6/group). ***p<0.001 compared to saline treatment by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* analysis. ***E.*** Effect of spinal level of injury on tactile withdrawal 10 weeks after injury in T3 complete transection (T3 Trans) and T11 complete transection (T11 Trans). N=4/group. **p<0.01, ***p<0.01 by one-way ANOVA followed by Tukey's *post-hoc* test. Data are expressed as mean ± SEM.

Figure 4. Cold allodynia develops in the forepaws after T3 severe SCI. ***A.*** T3 severe compression in response to the probe held at 13 °C, compared to sham control. ***B.*** T3 severe compression in response to the probe held at room temperature (RT). ***C.*** T3 complete transection in response to to the probe held at 13 °C, compared to naïve and sham control. ***D.*** T3 complete transection in response to the probe held at RT. Sensitivity to cold was assessed by withdrawal frequency in response to contact with at 13 °C Peltier probe. N=6-9/group. **p<0.01, ***p<0.001 compared to sham control and naive by two-way repeated measures ANOVA followed by Bonferroni *post-hoc* analysis. ***E.*** Core and forepaw surface temperature in naïve, sham control and rats with T3 complete transection 13 weeks following injury. N=8-9/group. No significant difference between groups by one way ANOVA. Data are expressed as mean ± SEM.

Figure 5. Alterations in CGRP expression and intensity of the IB4-binding population in C6-C8 spinal dorsal horn after severe SCI. Immunofluorescence microscopy was performed to detect CGRP in ***A.*** Naïve ***B.*** Sham control and ***C.*** T3 complete transected rats***. D.*** Pooled quantification of C6-C8 CGRP immunofluorescence intensity. IB4-binding in ***E.*** Naïve ***F.*** Sham control and ***G.*** T3 complete transected rats***. H.*** Pooled quantification of C6-C8 IB4 immunofluorescence intensity. Area of CGRP+ afferents and IB4-binding overlap in ***I.*** Naïve ***J.*** Sham control and ***K.*** T3 complete transected rats***. L.*** Pooled quantification of C6-C8 CGRP immunofluorescence and IB4-binding intensity. Quantification of immunofluorescence intensity is expressed as mean ± SEM. N=3-5/group. *p<0.01 compared to naïve sham control by one-way ANOVA. Scale bar= 200

μM. ***M***. GAP-43 levels were determined by immunoblot analysis in extracts of rat C6-C8 cervical dorsal horn 4 weeks after T3 complete transection. Blots were re-probed for β-actin as a loading control. Two representative rats from each group are shown. Equal amounts of cellular protein (20 μg) were loaded into each lane and subjected to SDS-PAGE. N=4/group. *p<0.05 by t-test compared to sham control. Results are expressed as mean ± SEM.

Figure 6. Increased IBA1 and GFAP expression in C6-C8 spinal dorsal horn after severe SCI. Immunofluorescence microscopy was performed to detect IBA1 in ***A.*** Naïve ***B.*** Sham control and ***C.*** T3 complete transected rats 4 weeks after injury***. D.*** Pooled quantification of C6-C8 IBA1 immunofluorescence intensity. GFAP expression in ***E.*** Naïve ***F.*** Sham control and ***G.*** T3 complete transected rats 4 weeks after injury***. H.*** Pooled quantification of C6-C8 CGRP immunofluorescence intensity. Quantification of immunofluorescence intensity is expressed as mean ± SEM. n=3-5/group. *p<0.01 compared to naïve sham control by one-way ANOVA. Scale bar= 200 μM. Iba1 and GFAP levels were determined by immunoblot analysis in extracts of rat C6-C8 cervical dorsal spinal cord ***I.*** 4 weeks and ***J.*** 8 weeks after T3 complete transection. Blots were re-probed for β-actin as a loading control. Two representative rats from each group are shown. Equal amounts of cellular protein (20 μg) were loaded into each lane and subjected to SDS-PAGE. N=4/group. *p<0.05, **p<0.01 by t-test compared to sham control. Data are expressed as mean ± SEM.

Figure 7. Increased connexin-43 expression in C6-C8 spinal dorsal horn following T3 complete transection. Connexin-43 at ***A***. 4 weeks after SCI and ***B.*** 8 weeks after SCI Connexin-43 were determined by immunoblot anlaysis. Blots were re-probed for β-neuronal tubulin (Tuj1) as a loading control. Two representative rats from each group are shown. Equal amounts of cellular protein (20 μg) were loaded into each lane and subjected to SDS-PAGE. **C.** Quantification of immunoblot results is expressed as mean ± SEM. N=4/group. *p<0.05, **p<0.01 by t-test compared to sham control. Dual labeling immunofluorescence microscopy was performed to detect ***D.*** connexin-43 (CX-43) and GFAP, ***E.*** CX-43 and NeuN, and ***F.*** CX-43 and IBA1 in T3 complete transected rats 4

weeks after injury. Z-stack images generated with 0.57 µM optical sections. Scale bar = 10 µM.

Figure 8. SGCs become active in DRGs several segments rostral to injury site. ***A***. GFAP levels and ***B.*** Connexin-43 were determined by immunoblot analysis in extracts of rat C6-C8 DRGs 4 weeks after injury. Blots were re-probed for β-neuronal tubulin (Tuji) as a loading control. Two representative rat DRGs are shown. Equal amounts of cellular protein (10 µg) were loaded into each lane and subjected to SDS-PAGE. Quantification of immunoblot results is expressed as mean ± SEM. N=3-9/group. *p<0.05, **p<0.01 by t-test compared to sham control. Dual labeling immunofluorescence microscopy was performed to detect ***C.*** Connexin-43 (green), GFAP (red) and NeuN (cyan) expression in a C7 DRG from a sham and T3 transected rat, 4 weeks after injury. Connexin-43 is primarily expressed in the perineuronal region surrounding NeuN, and co-localizes with GFAP following complete transection. ***D.*** Maximum projection z-stack of triple labeling immunofluorescence including connexin-43, GFAP and NeuN expression in the C7 DRG collected from naïve, sham and T3 complete transected rats. ***E.*** Pooled quantification of the percent area of connexin-43 immunoreactivity in C6-C8 DRG. **F.** Quantification of the percent area of connexin-43 immunoreactivity separated into C6-C8 DRGs collected from rats 4 weeks following T3 complete transection. Data are expressed as mean ± SEM. n=3-5/group. *p<0.05, **p<0.01 compared to C6 by one-way ANOVA followed by Tukey's *post-hoc* test.

Figure 9. Blocking connexin-43 alleviates established allodynia after T3 complete transection ***A.*** Effects of carbenoxolone (25 µg IT) or glycyrrhyzic acid (25 µg IT) on tactile withdrawal threshold 4 weeks after T3 complete transection. ***B.*** Effects of Gap27-scramble, Gap27 or Gap19 (13 µg IT) on forepaw tactile withdrawal threshold 4 weeks after T3 complete transection. ***C.*** Effects of Gap27 (13 µg IT) on forepaw withdrawal threshold in naïve or sham control rats. ***D.*** Effects of Gap26 (13 µg IT) or vehicle control on forepaw withdrawal threshold 4 weeks after complete transection. Forepaw tactile withdrawal threshold is expressed as mean ± SEM. N=6-7/treatment group.

*p<0.05, **p<0.01 compared to saline treatment by two-way ANOVA followed by Bonferroni *post-hoc* test.

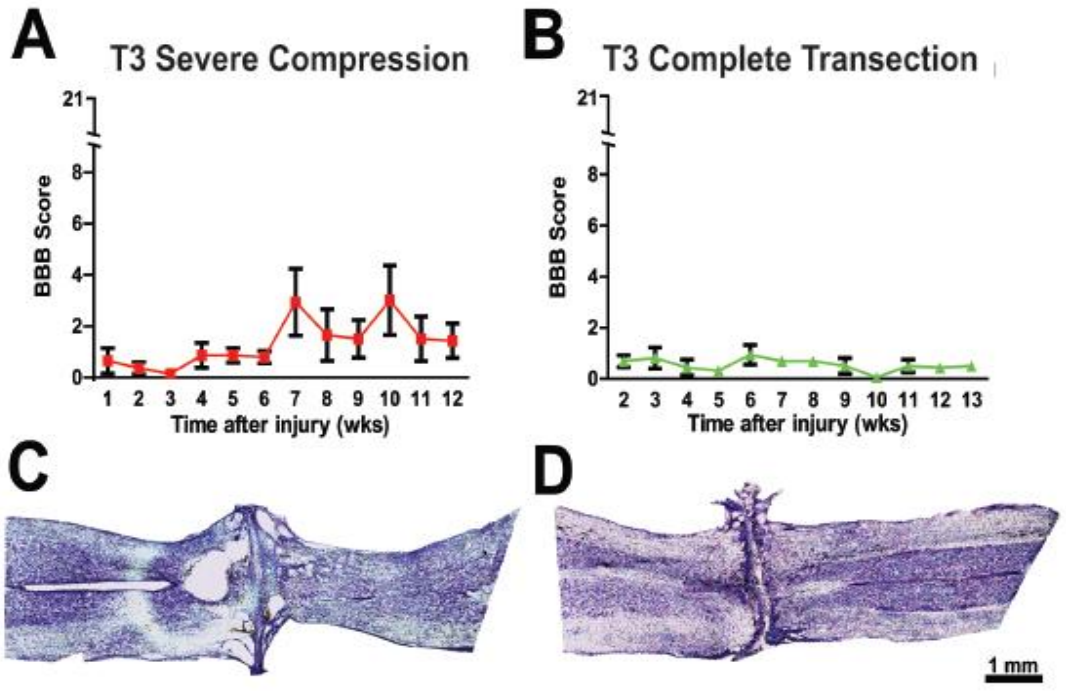
A
T3 Severe Compression
BBB Score
21
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12
Time after injury (wks)
B
T3 Complete Transection
BBB Score
21
8
6
4
2
0
2 3 4 5 6 7 8 9 10 11 12 13
Time after injury (wks)
C
D
1 mm

Figure 1

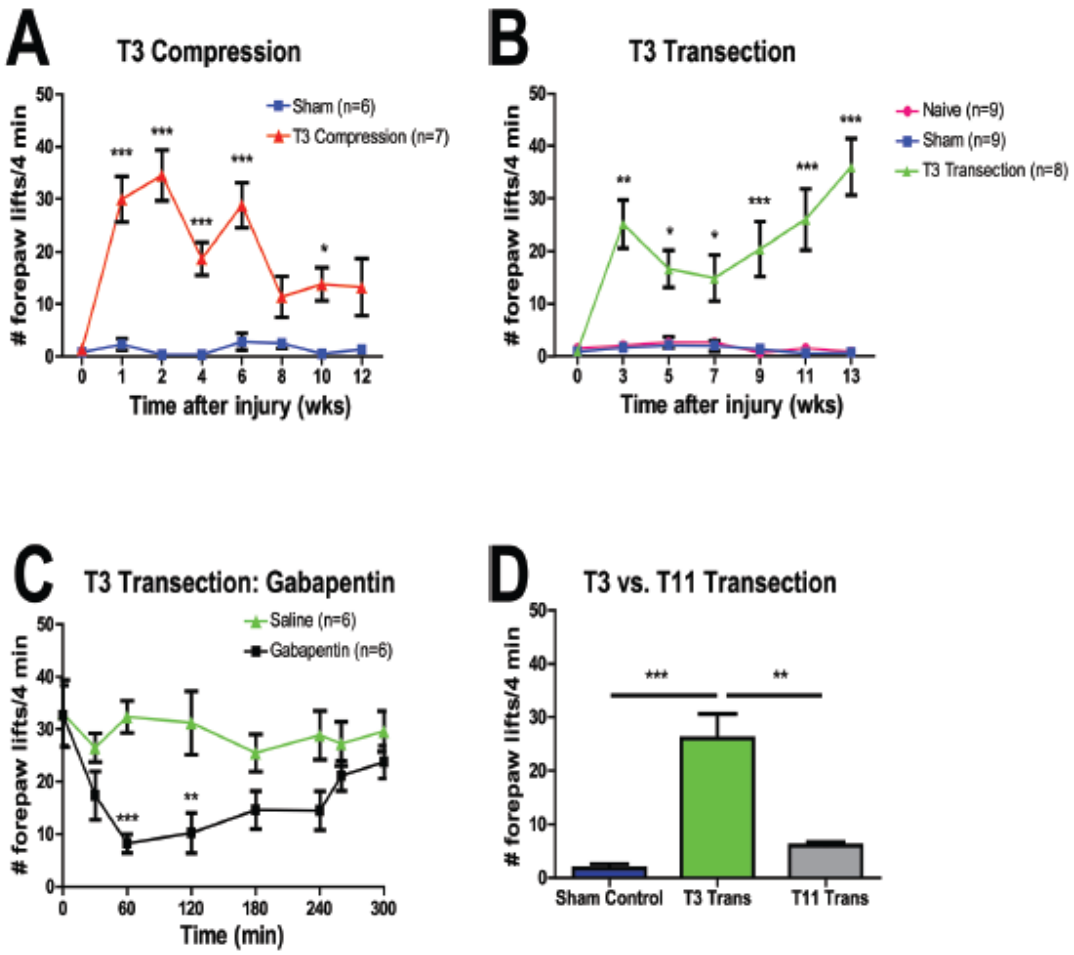
A
T3 Compression
Sham (n=6)
T3 Compression (n=7)
forepaw lifts/4 min
Time after injury (wks)
B
T3 Transection
Naive (n=9)
Sham (n=9)
T3 Transection (n=8)
forepaw lifts/4 min
Time after injury (wks)
C
T3 Transection: Gabapentin
Saline (n=6)
Gabapentin (n=6)
forepaw lifts/4 min
Time (min)
D
T3 vs. T11 Transection
forepaw lifts/4 min
Sham Control
T3 Trans
T11 Trans

Figure 2

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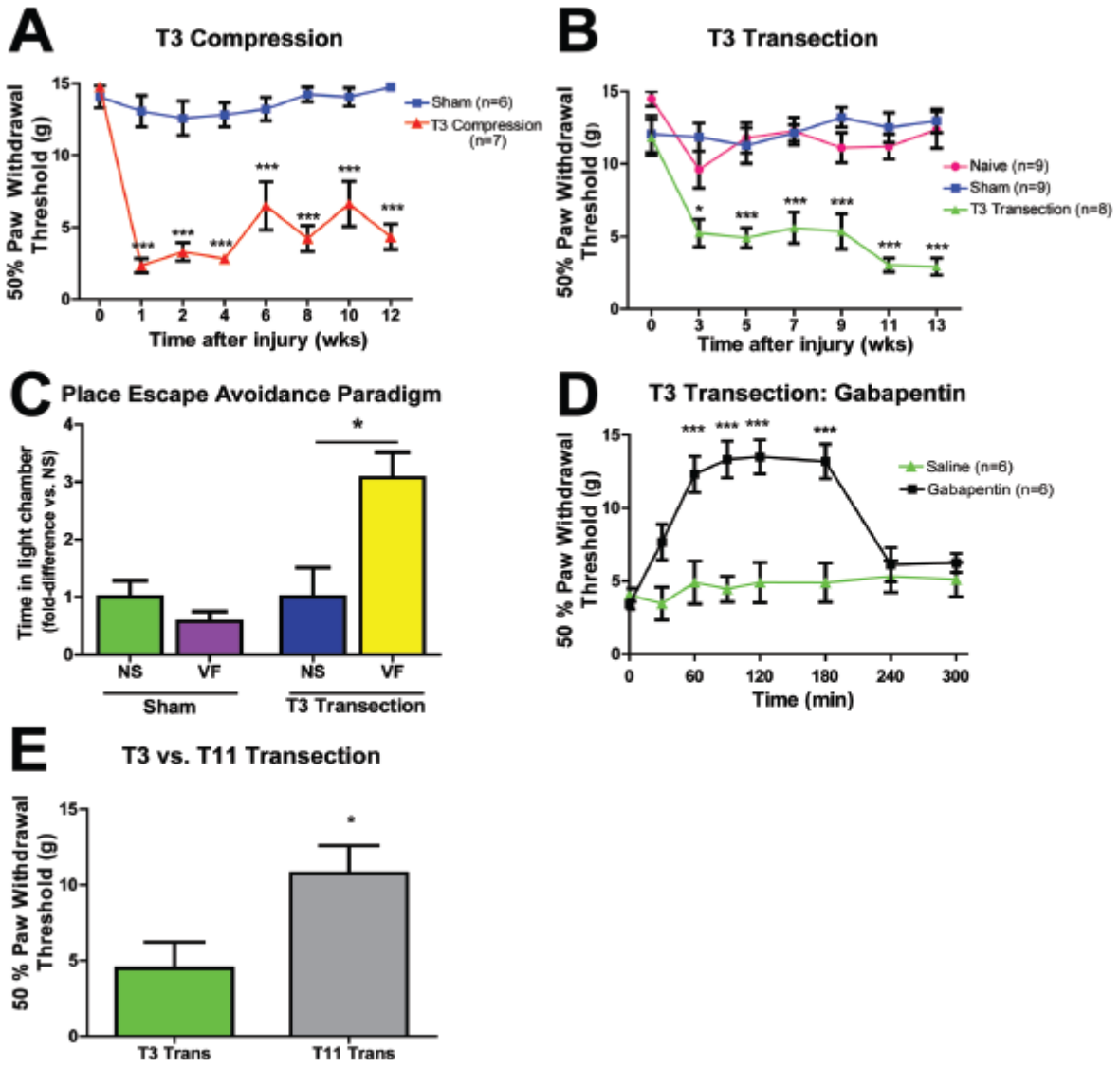
A
T3 Compression
50% Paw Withdrawal Threshold (g)
Time after injury (wks)
Sham (n=6)
T3 Compression (n=7)
B
T3 Transection
50% Paw Withdrawal Threshold (g)
Time after injury (wks)
Naive (n=9)
Sham (n=9)
T3 Transection (n=8)
C
Place Escape Avoidance Paradigm
Time in light chamber (fold-difference vs. NS)
NS
VF
NS
VF
Sham
T3 Transection
D
T3 Transection: Gabapentin
50 % Paw Withdrawal Threshold (g)
Time (min)
Saline (n=6)
Gabapentin (n=6)
E
T3 vs. T11 Transection
50 % Paw Withdrawal Threshold (g)
T3 Trans
T11 Trans

Figure 3

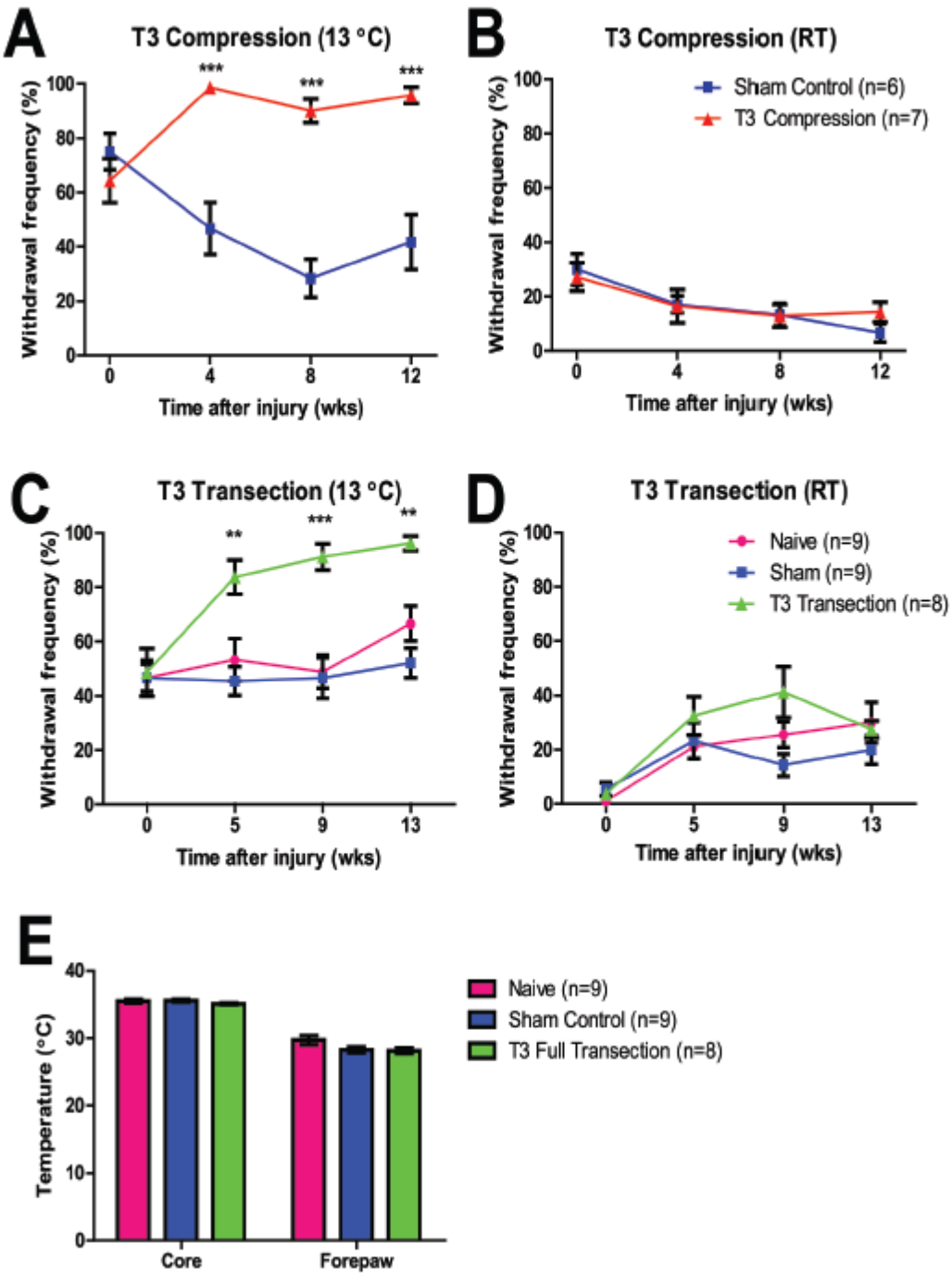
A
T3 Compression (13 °C)
Withdrawal frequency (%)
Time after injury (wks)
B
T3 Compression (RT)
Sham Control (n=6)
T3 Compression (n=7)
Withdrawal frequency (%)
Time after injury (wks)
C
T3 Transection (13 °C)
Withdrawal frequency (%)
Time after injury (wks)
D
T3 Transection (RT)
Naive (n=9)
Sham (n=9)
T3 Transection (n=8)
Withdrawal frequency (%)
Time after injury (wks)
E
Temperature (°C)
Core
Forepaw
Naive (n=9)
Sham Control (n=9)
T3 Full Transection (n=8)

Figure 4

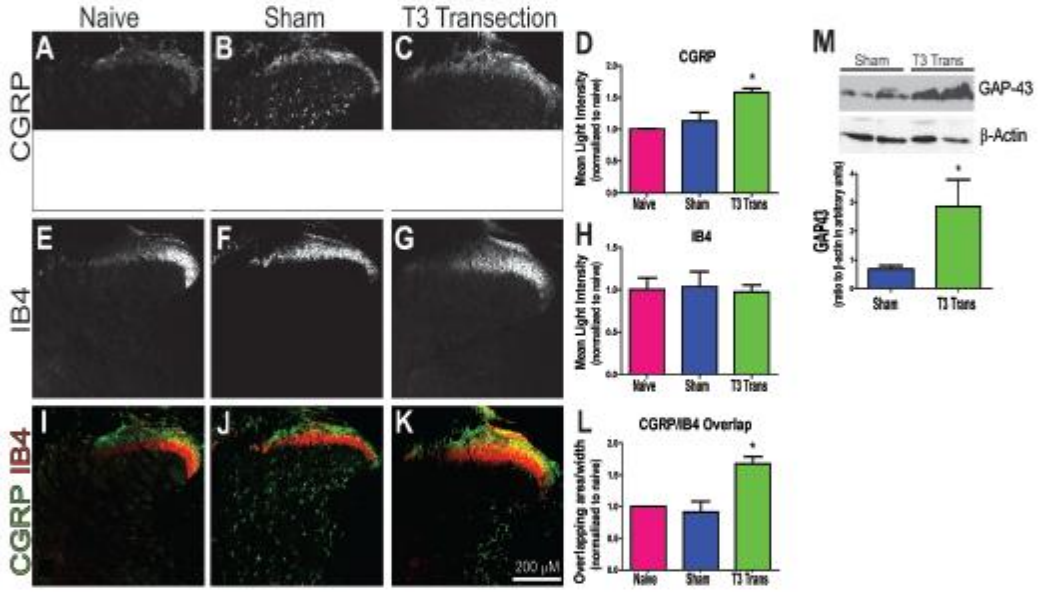
Naive
Sham
T3 Transection
CGRP
IB4
CGRP IB4
A
B
C
D
E
F
G
H
I
J
K
L
M
200 μM
CGRP
IB4
CGRP/IB4 Overlap
Mean Light Intensity (normalised to naive)
Overlapping area/width (normalised to naive)
Naive
Sham
T3 Trans
Sham
T3 Trans
GAP-43
β-Actin
GAP43 (ratio to β-actin in arbitrary units)

Figure 5

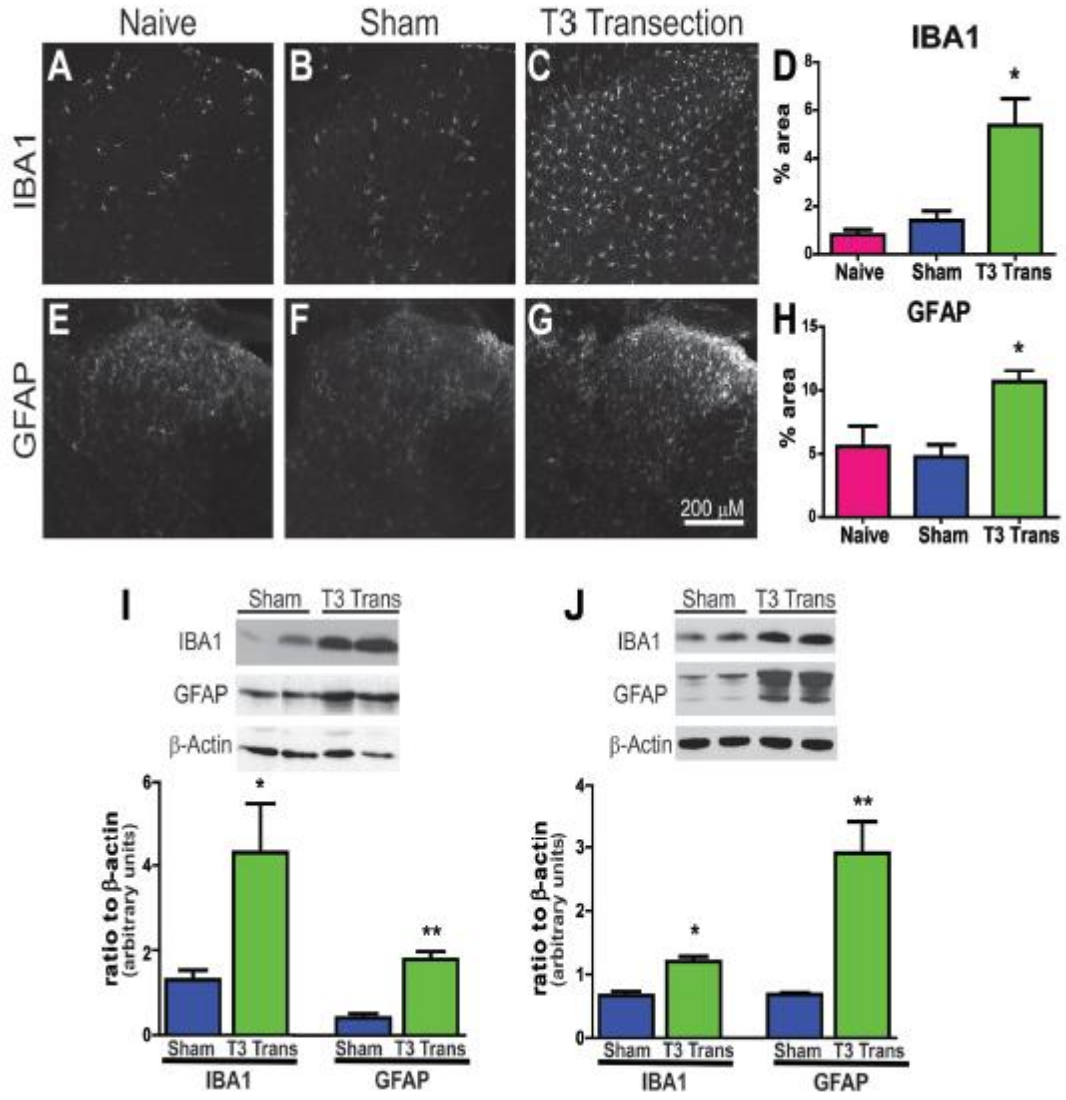
Naive
Sham
T3 Transection
IBA1
GFAP
A
B
C
D
E
F
G
H
I
J
IBA1
% area
Naive
Sham
T3 Trans
GFAP
200 µM
Sham
T3 Trans
β-Actin
ratio to β-actin
(arbitrary units)
IBA1
GFAP

Figure 6

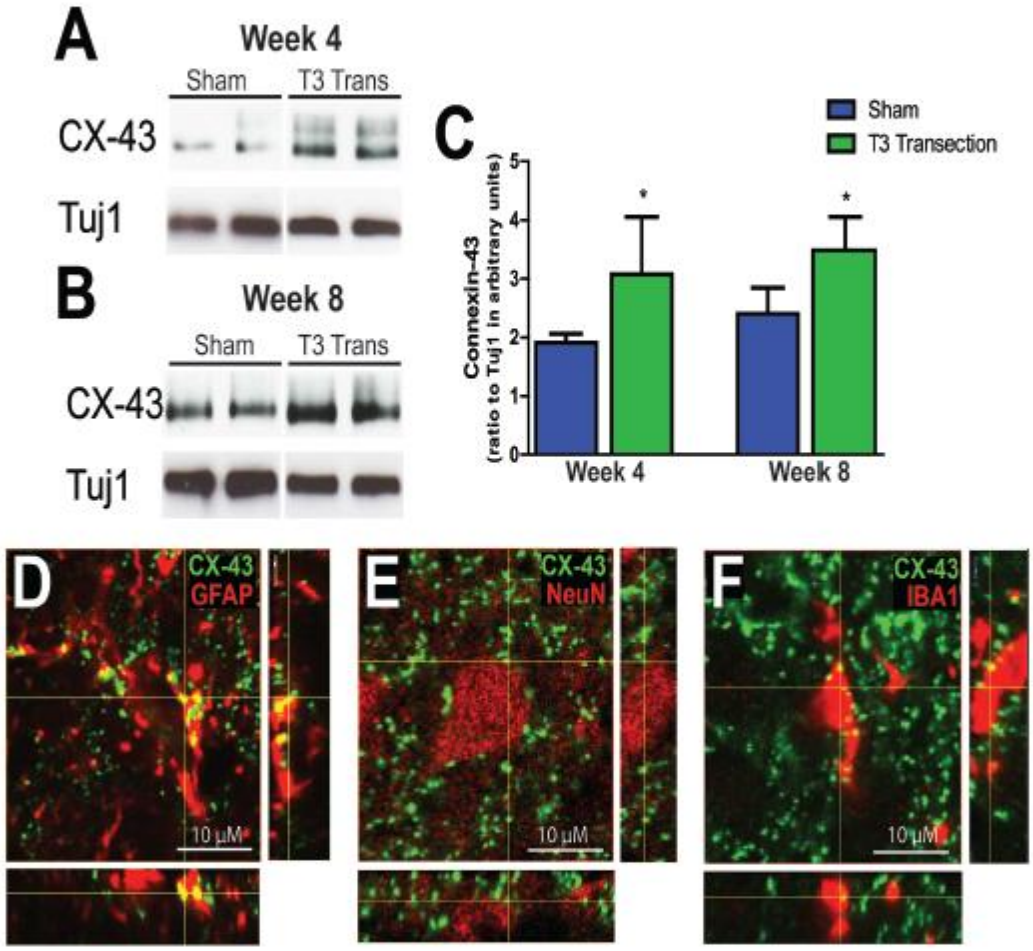
A
Week 4
Sham
T3 Trans
CX-43
Tuj1
B
Week 8
Sham
T3 Trans
CX-43
Tuj1
C
Sham
T3 Transection
Connexin-43
(ratio to Tuj1 in arbitrary units)
0
1
2
3
4
5
*
*
Week 4
Week 8
D
CX-43
GFAP
10 µM
E
CX-43
NeuN
10 µM
F
CX-43
IBA1
10 µM

Figure 7

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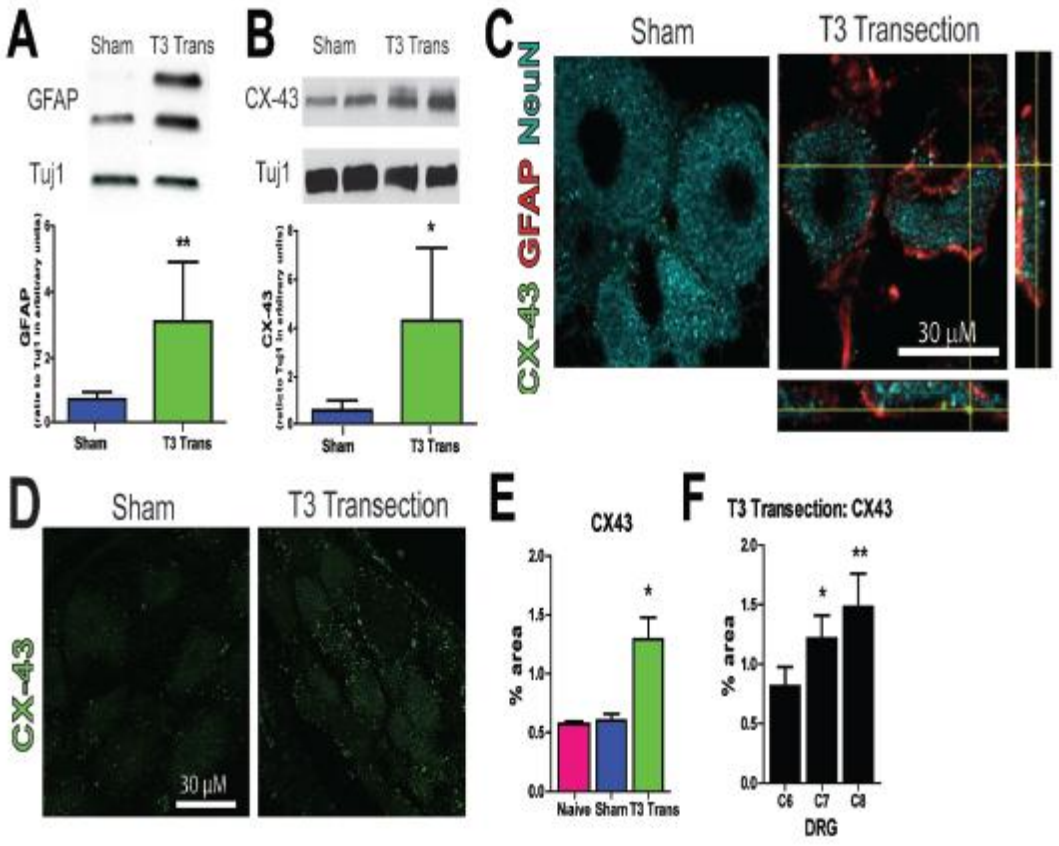
A
Sham
T3 Trans
GFAP
Tuj1
GFAP (ratio to Tuj1 in arbitrary units)
Sham
T3 Trans
B
Sham
T3 Trans
CX-43
Tuj1
CX-43 (ratio to Tuj1 in arbitrary units)
Sham
T3 Trans
C
Sham
T3 Transection
CX-43 GFAP NeuN
30 µM
D
Sham
T3 Transection
CX-43
30 µM
E
CX43
% area
Naive Sham T3 Trans
F
T3 Transection: CX43
% area
C6 C7 C8
DRG

Figure 8

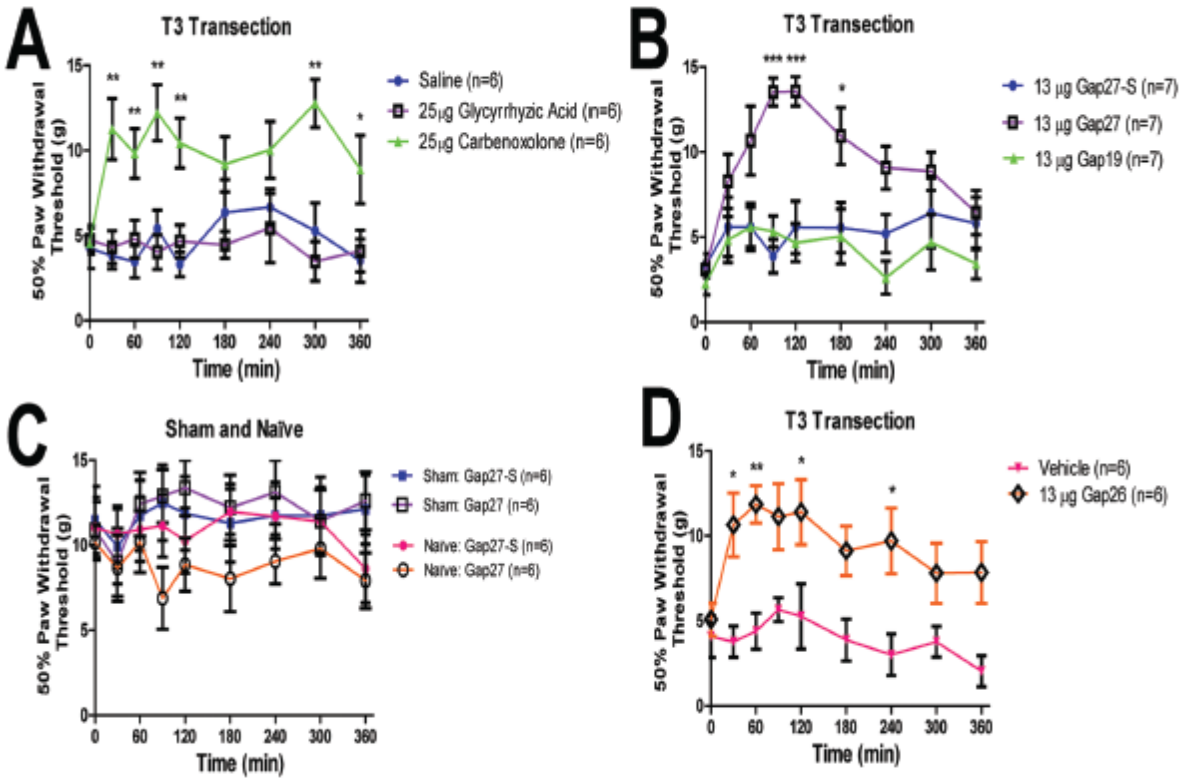
A
T3 Transection
50% Paw Withdrawal Threshold (g)
Time (min)
Saline (n=6)
25μg Glycyrrhyzic Acid (n=6)
25μg Carbenoxolone (n=6)
B
T3 Transection
50% Paw Withdrawal Threshold (g)
Time (min)
13 μg Gap27-S (n=7)
13 μg Gap27 (n=7)
13 μg Gap19 (n=7)
C
Sham and Naïve
50% Paw Withdrawal Threshold (g)
Time (min)
Sham: Gap27-S (n=6)
Sham: Gap27 (n=6)
Naive: Gap27-S (n=6)
Naive: Gap27 (n=6)
D
T3 Transection
50% Paw Withdrawal Threshold (g)
Time (min)
Vehicle (n=6)
13 μg Gap26 (n=6)

Figure 9

MECHANISMS ASSOCIATED WITH PERSISTENT PAIN STATES AFTER SEVERE RODENT SPINAL CORD INJURY

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Human spinal cord injury (SCI) is frequently associated with chronic pain that is severe and refractory to medical therapy. Most rodent models used to assess pain outcomes in SCI apply moderate injuries to lower thoracic spinal levels, whereas the majority of human lesions are severe in degree and occur at cervical or upper thoracic levels. To better model and understand mechanisms associated with chronic pain after SCI, we subjected adult rats to T3 severe compression or complete transection lesions, and examined pain-related behaviors for three months. Within one week of injury, rats developed consistent forepaw pain-related behaviors including increased spontaneous lifts, tactile allodynia and cold sensitivity that persisted for three months. Spontaneous lifts and tactile allodynia were attenuated by gabapentin, suggesting that these behaviors reflect development of pain. Spinal level of injury was relevant: rats with T11 severe SCI did not exhibit forepaw pain-related behaviors. Immunoblotting and immunofluorescence of C6-C8 spinal dorsal horn, reflecting sensory innervation of the forepaw, revealed increased IBA1, GFAP and connexin-43 expression, indicative of gliopathy. Notably, satellite cells in C6-C8 DRGs also exhibited increased GFAP and connexin-43 expression, suggesting ongoing peripheral sensitization. Intrathecal administration of carbenoxolone, a gap junction inhibitor, and Gap26 and Gap27, peptide inhibitors of connexin-43, directly to the C6-C8 spinal segments ameliorated established tactile allodynia after severe SCI, confirming contribution of connexin-43 to the maintenance of neuropathic pain following severe SCI. Collectively, severe T3 SCI successfully models persistent pain states and could constitute a useful model system for examining candidate translational therapies for treatment of at-level SCI pain.